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(54) Title: FOUR-COLOR DNA SEQUENCING BY SYNTHESIS USING CLEAVABLE FLUORESCNT NUCLEOTIDE REVERSIBLE TERMINATORS

(57) Abstract: This invention provides a process for sequencing single- stranded DNA by employing a nanopore and modified nucleotides.

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Exhibit 1

FOUR-COLOR DNA SEQUENCING BY SYNTHESIS USING CLEAVABLE FLOURESCENT NUCLEOTIDE REVERSIBLE TERMINATORS

The invention disclosed herein was made with government support NIH grant P50-HG002806. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various publications are referenced in parentheses by number. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Background of the Invention

DNA sequencing is driving genomics research and discovery. The completion of the Human Genome Project has set the stage for screening genetic mutations to identify disease genes on a genome-wide scale (1). Accurate high-throughput DNA sequencing methods are needed to explore the complete human genome sequence for applications in clinical medicine and health care. To overcome the limitations of the current electrophoresis-based sequencing technology (2-5), a variety of new DNA-sequencing methods have been investigated. Such approaches include sequencing by

hybridization (6), mass spectrometry based sequencing (7-9), sequence-specific detection of single-stranded DNA using engineered nanopores (10) and sequencing by ligation (11). More recently, DNA sequencing by synthesis (SBS) approaches such as pyrosequencing (12), sequencing of single DNA molecules (13) and polymerase colonies (14) have been widely explored.

The concept of DNA sequencing by synthesis (SBS) was revealed in 1988 with an attempt to sequence DNA by detecting the pyrophosphate group that is generated when a nucleotide is incorporated in a DNA polymerase reaction (15). Pyrosequencing which was developed based on this concept and an enzymatic cascade has been explored for genome sequencing (16). However, there are inherent difficulties in this method for determining the number of incorporated nucleotides in homopolymeric regions of the template. Additionally, each of the four nucleotides needs to be added and detected separately, which increases the overall detection time. The accumulation of un-degraded nucleotides and other components could also lower the accuracy of the method when sequencing a long DNA template. It is thus desirable to have a simple method to directly detect a reporter group attached to the nucleotide that is incorporated into a growing DNA strand in the polymerase reaction rather than relying on a complex enzymatic cascade. The SBS scheme based on fluorescence detection coupled with a chip format has the potential to markedly increase the throughput of DNA sequencing projects. Consequently, several groups have investigated such a system with an aim to

construct an ultra high-throughput DNA sequencing method (17-18). Thus far, no complete success of using such a system to unambiguously sequence DNA has been published.

Previous work in the literature exploring the SBS method is mostly focused on designing and synthesizing a cleavable chemical moiety that is linked to a fluorescent dye to cap the 3'-OH group of the nucleotides (19-21). The rationale is that after the fluorophore is removed, the 3'-OH would be regenerated to allow subsequent nucleotide addition. However, no success has been reported for the incorporation of such a nucleotide with a cleavable fluorescent dye on the 3' position by DNA polymerase into a growing DNA strand. The reason is that the 3' position on the deoxyribose is very close to the amino acid residues in the active site of the polymerase, and the polymerase is therefore sensitive to modification in this area of the ribose ring, especially with a large fluorophore (22).

Summary of the Invention

This invention provides a method for determining the sequence of a DNA comprising performing the following steps for each residue of the DNA to be sequenced:

(a) contacting the DNA with a DNA polymerase in the presence of (i) a primer and (ii) four nucleotide analogues under conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (1) the nucleotide analogues consist of an analogue of dGTP, an analogue of dCTP, an analogue of dTTP or dUTP, and an analogue of dATP, (2) each nucleotide analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, and analogues thereof, (ii) a deoxyribose, (iii) a moiety cleavably linked to the 3'-oxygen of the deoxyribose and (iv) a unique label cleavably linked to the base, so that a nucleotide analogue complementary to the residue being sequenced is incorporated into the DNA by the DNA polymerase, and (3) each of the four analogues has a unique label which is different than the unique labels of the other three analogues;

(b) removing unbound nucleotide analogues;

(c) again contacting the DNA with a DNA polymerase in the presence of (i) a primer and (ii) four reversible terminators under

conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (1) the reversible terminators consist of an analogue of dGTP, an analogue of dCTP, an analogue of dTTP or dUTP, and an analogue of dATP, (2) each nucleotide analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, and analogues thereof, which base does not have a unique label bound thereto, (ii) a deoxyribose, and (iii) a moiety cleavably linked to the 3'-oxygen of the deoxyribose;

(d) removing unbound reversible terminators;

(e) determining the identity of the nucleotide analogue incorporated in step (a) via determining the identity of the corresponding unique label, with the proviso that step (e) can either precede step (c) or follow step (d); and

(f) following step (e), except with respect to the final DNA residue to be sequenced, cleaving from the incorporated nucleotide analogues the unique label, if applicable, and the moiety linked to the 3'-oxygen atom of the deoxyribose,

thereby determining the sequence of the DNA.

This invention also provides a kit for performing the method of claim 1, comprising, in separate compartments,

(a) nucleotide analogues of (i) GTP, (ii) ATP, (iii) CTP and (iv) TTP or UTP, wherein each analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, or an analogue thereof, (ii) a deoxyribose, (iii) a cleavable moiety bound to the 3'-oxygen of the deoxyribose and (iv) a unique label bound to the base via a cleavable linker,

(b) reversible terminators comprising a nucleotide analogue of (i) GTP, (ii) ATP, (iii) CTP and (iv) TTP or UTP, wherein each analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, or an analogue thereof, which base does not have a unique label bound thereto, (ii) a deoxyribose, and (iii) a cleavable moiety bound to the 3'-oxygen of the deoxyribose;

(c) reagents suitable for use in DNA polymerization; and

(d) instructions for use.

Brief Description of the Figures

Figure 1. A chip is constructed with immobilized DNA templates that are able to self-prime for initiating the polymerase reaction. Four nucleotide analogues are designed such that each is labeled with a unique fluorescent dye on the specific location of the base through a cleavable linker, and a small chemically reversible moiety (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only the nucleotide analogue complementary to the next nucleotide on the template is incorporated by polymerase on each spot of the chip (step 1). A 4 color fluorescence imager is used to image the surface of the chip, and the unique fluorescence emission from the specific dye on the nucleotide analogues on each spot of the chip will yield the identity of the nucleotide (step 2). After imaging, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess ddNTPs and DNA polymerase to avoid interference with the next round of synthesis or by 3'-O-allyl-dNTPs to synchronize the incorporation (step 3). The dye moiety and the R protecting group will be removed to generate a free 3'-OH group with high yield (step 4). The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (step 5).

Figure 2. Structures of 3'-O-allyl-dCTP-allyl-Bodipy-FL-510 ($\lambda_{abs\ (max)}$ = 502 nm; $\lambda_{em\ (max)}$ = 510 nm), 3'-O-

allyl-dUTP-allyl-R6G ($\lambda_{abs\ (max)}$ = 525 nm; $\lambda_{em\ (max)}$ = 550 nm), 3'-O-allyl-dATP-allyl-ROX ($\lambda_{abs\ (max)}$ = 585 nm; $\lambda_{em\ (max)}$ = 602 nm), and 3'-O-allyl-dGTP-allyl-Bodipy-650 ($\lambda_{abs\ (max)}$ = 630 nm; $\lambda_{em\ (max)}$ = 650 nm).

Figure 3. The polymerase extension scheme (left) and MALDI-TOF MS spectra of the four consecutive extension products and their deallylated products (right). Primer extended with 3'-O-allyl-dUTP-allyl-R6G (**1**), and its deallylated product **2**; Product **2** extended with 3'-O-allyl-dGTP-allyl-Bodipy-650 (**3**), and its deallylated product **4**; Product **4** extended with 3'-O-allyl-dATP-allyl-ROX (**5**), and its deallylated product **6**; Product **6** extended with 3'-O-allyl-dCTP-allyl-Bodipy-FL-510 (**7**), and its deallylated product **8**. After 30 seconds of incubation with the palladium/TPPTS cocktail at 70°C, deallylation is complete with both the fluorophores and the 3'-O-allyl groups cleaved from the extended DNA products.

Figure 4. DNA extension reaction performed in solution phase to characterize the four different chemically cleavable fluorescent nucleotide analogues (3'-O-allyl-dUTP-allyl-R6G, 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dCTP-allyl-Bodipy-FL-510). After each extension reaction, the DNA extension product is purified by HPLC for MALDI-TOF MS measurement to verify that it is the correct extension product. Pd-catalyzed deallylation reaction is performed to produce a DNA product that is used as a primer for the next DNA extension reaction.

Figure 5. Preparation of azide-functionalized glass chip through a PEG linker for the immobilization of alkyne labeled self-priming DNA template for SBS.

Figure 6. **(A)** Reaction scheme of SBS on a chip using four chemically cleavable fluorescent nucleotides. **(B)** The scanned 4-color fluorescence images for each step of SBS on a chip: (1) incorporation of 3'-O-allyl-dGTP-allyl-Cy5; (2) cleavage of allyl-Cy5 and 3'-allyl group; (3) incorporation of 3'-O-allyl-dATP-allyl-ROX; (4) cleavage of allyl-ROX and 3'-allyl group; (5) incorporation of 3'-O-allyl-dUTP-allyl-R6G; (6) cleavage of allyl-R6G and 3'-allyl group; (7) incorporation of 3'-O-allyl-dCTP-allyl-Bodipy-FL-510; (8) cleavage of allyl-Bodipy-FL-510 and 3'-allyl group; images (9) to (25) are similarly produced. **(C)** A plot (4-color sequencing data) of raw fluorescence emission intensity at the four designated emission wavelength of the four chemically cleavable fluorescent nucleotides.

Figure 7. Structures of 3'-O-allyl-dATP, 3'-O-allyl-dCTP, 3'-O-allyl-dGTP, and 3'-O-allyl-dTTP.

Figure 8. **(A)** 4-color DNA sequencing raw data with our sequencing by synthesis chemistry using a template containing two homopolymeric regions. The individual base (A, T, C, G), the 10 repeated A's and the 5 repeated A's are clearly identified. The small groups of peaks between the identified bases are fluorescent background from the DNA chip, which does not build up

as the cycle continues. (**B**) The pyrosequencing data of the same DNA template containing the homopolymeric regions (10 T's and 5 T's). The first 4 individual bases are clearly identified. The two homopolymeric regions (10 A's) and (5 A's) produce two large peaks, making it very difficult to determine the exact sequence from the data.

Figure 9. Single base extension reaction and MALDI-TOF MS of 3'-*O*-Allyl-dUTP-allyl-R6G.

Figure 10. Single base extension reaction and MALDI-TOF MS of 3'-*O*-Allyl-dATP-allyl-ROX (39).

Figure 11. Single base extension reaction and MALDI-TOF MS of 3'-*O*-Allyl-dGTP-allyl-Bodipy-650 (43) and 3'-*O*-allyl-dGTP-allyl-Cy5 (44).

Detailed Description of the Invention

Terms

As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.

A	-	Adenine;
C	-	Cytosine;
DNA	-	Deoxyribonucleic acid;
G	-	Guanine;
RNA	-	Ribonucleic acid;
T	-	Thymine; and
U	-	Uracil.

"Nucleic acid" shall mean any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form nucleic acid molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA).

"Type" of nucleotide refers to A, G, C, T or U.

"Mass tag" shall mean a molecular entity of a predetermined size which is capable of being attached by a cleavable bond to another entity.

"Solid substrate" shall mean any suitable medium present in the solid phase to which an antibody or an agent may be affixed.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Embodiments of the Invention

This invention provides a method for determining the sequence of a DNA comprising performing the following steps for each residue of the DNA to be sequenced:

(a) contacting the DNA with a DNA polymerase in the presence of (i) a primer and (ii) four nucleotide analogues under conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (1) the nucleotide analogues consist of an analogue of dGTP, an analogue of dCTP, an analogue of dTTP or dUTP, and an analogue of dATP, (2) each nucleotide analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, and analogues thereof, (ii) a deoxyribose, (iii) a moiety cleavably linked to the 3'-oxygen of the deoxyribose and (iv) a unique label cleavably linked to the base, so that a nucleotide analogue complementary to the residue being sequenced is incorporated into the DNA by the DNA polymerase, and (3) each of the four analogues has a unique label which is different than the unique labels of the other three analogues;

(b) removing unbound nucleotide analogues;

(c) again contacting the DNA with a DNA polymerase in the presence of (i) a primer

and (ii) four reversible terminators under conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (1) the reversible terminators consist of an analogue of dGTP, an analogue of dCTP, an analogue of dTTP or dUTP, and an analogue of dATP, (2) each nucleotide analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, and analogues thereof, which base does not have a unique label bound thereto, (ii) a deoxyribose, and (iii) a moiety cleavably linked to the 3'-oxygen of the deoxyribose;

(d) removing unbound reversible terminators;

(e) determining the identity of the nucleotide analogue incorporated in step (a) via determining the identity of the corresponding unique label, with the proviso that step (e) can either precede step (c) or follow step (d); and

(f) following step (e), except with respect to the final DNA residue to be sequenced, cleaving from the incorporated nucleotide analogues the unique label, if applicable, and the moiety linked to the 3'-oxygen atom of the deoxyribose,

thereby determining the sequence of the DNA.

This invention also provides the instant method, wherein step (e) is performed before step (c).

This invention also provides the instant method, wherein the moiety cleavably linked to the 3'-oxygen of the deoxyribose is chemically cleavable or photocleavable. This invention also provides the instant method, wherein the moiety cleavably linked to the 3'-oxygen of the deoxyribose in the nucleotide analogs of step (a) is an allyl moiety or a 2-nitrobenzyl moiety.

This invention also provides the instant method, wherein the moiety cleavably linked to the 3'-oxygen of the deoxyribose in the reversible terminators of step (c) is an allyl moiety or a 2-nitrobenzyl moiety.

This invention also provides the instant method, wherein the unique label is bound to the base via a chemically cleavable or photocleavable linker.

This invention also provides the instant method, wherein the unique label bound to the base via a cleavable linker is a dye, a fluorophore, a chromophore, a combinatorial fluorescence energy transfer tag, a mass tag, or an electrophore.

This invention also provides the instant method, wherein the moiety is chemically cleavable with $Na_2PdCl_4/P(PhSO_3Na)_3$. This invention also provides the instant method, wherein the linker is chemically cleavable with $Na_2PdCl_4/P(PhSO_3Na)_3$.

This invention also provides the instant method, wherein the primer is a self-priming moiety.

This invention also provides the instant method, wherein the DNA is bound to a solid substrate. This invention also provides the instant method, wherein the DNA is bound to the solid substrate via 1,3-dipolar azide-alkyne cycloaddition chemistry. This invention also provides the instant method, wherein the DNA is bound to the solid substrate via a polyethylene glycol molecule. This invention also provides the instant method, wherein the DNA is alkyne-labeled. This invention also provides the instant method, wherein the DNA is bound to the solid substrate via a polyethylene glycol molecule and the solid substrate is azide-functionalized. This invention also provides the instant method, wherein the DNA is immobilized on the solid substrate via an azido linkage, an alkynyl linkage, or biotin-streptavidin interaction.

This invention also provides the instant method, wherein the solid substrate is in the form of a chip, a bead, a well, a capillary tube, a slide, a wafer, a filter, a fiber, a porous media, or a column. This invention also provides the instant method, wherein the solid substrate is gold, quartz, silica, plastic, glass, diamond, silver, metal, or polypropylene. This invention also provides the instant method, wherein the solid substrate is porous.

This invention also provides the instant method, wherein about 1000 or fewer copies of the DNA are bound to the solid substrate. This invention also

provides the instant invention wherein $1x10^7$, $1x10^6$ or $1x10^4$ or fewer copies of the DNA are bound to the solid substrate.

This invention also provides the instant method, wherein the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Cy5, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP- allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G. This invention also provides the instant method, wherein the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-FL-510, 3'-O-allyl-dCTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G. This invention also provides the instant method, wherein the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

It is understood that in other embodiments the nucleotide analogues are photocleavable. For example, 2-nitrobenzyl can replace any of the allyl moieties in the analogues described herein. For example, 3'-O-2-nitrobenzyl-dGTP-allyl-Bodipy-650, 3'-O-2-nitrobenzyl-dGTP-2-nitrobenzyl-Bodipy-650, 3'-O-ally-dGTP-2-nitrobenzyl-Bodipy-650. One of skill in the art would recognize various other chemically cleavable or photochemically cleavable moieties or linkers that can be used in place of the examples described herein. Additionally, the unique labels may also be varied, and the examples set forth herein are non-limiting. In an embodiment UV light is used to photochemically

cleave the photochemically cleavable linkers and moieties.

This invention also provides the instant method, wherein the reversible terminators in step (c) are 3'-O-allyl-dGTP, 3'-O-allyl-dCTP, 3'-O-allyl-dATP and 3'-O-allyl-dUTP. This invention also provides the instant method, wherein the reversible terminators in step (c) are 3'-O-2-nitrobenzyl-dGTP, 3'-O-2-nitrobenzyl-dCTP, 3'-O-2-nitrobenzyl-dATP and 3'-O-2-nitrobenzyl-dUTP. In an embodiment the reversible terminator is incorporated into the growing strand of DNA.

This invention also provides the instant method, wherein the DNA polymerase is a 9°N polymerase or a variant thereof. DNA polymerases which can be used in the instant invention include, for example E.Coli DNA polymerase I, Bacteriophage T4 DNA polymerase, Sequenase™, Taq DNA polymerase and 9°N polymerase (exo-) A485L/Y409V.RNA polymerases which can be used in the instant invention include, for example, Bacteriophage SP6, T7 and T3 RNA polymerases.

This invention also provides the instant method, wherein the DNA is bound to the solid substrate via a polyethylene glycol molecule and the solid substrate is azide-functionalized or the DNA is immobilized on the solid substrate via an azido linkage, an alkynyl linkage, or biotin-streptavidin interaction; wherein (i) the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Cy5, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP- allyl-ROX and 3'-O-allyl-

dUTP-allyl-R6G, (ii) the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-FL-510, 3'-O-allyl-dCTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G, or (iii) the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G; and wherein the reversible terminators in step (c) are 3'-O-allyl-dGTP, 3'-O-allyl-dCTP, 3'-O-allyl-dATP and 3'-O-allyl-dUTP.

This invention also provides a kit for performing the instant method comprising, in separate compartments,

(a) nucleotide analogues of (i) GTP, (ii) ATP, (iii) CTP and (iv) TTP or UTP, wherein each analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, or an analogue thereof, (ii) a deoxyribose, (iii) a cleavable moiety bound to the 3'-oxygen of the deoxyribose and (iv) a unique label bound to the base via a cleavable linker,

(b) reversible terminators comprising a nucleotide analogue of (i) GTP, (ii) ATP, (iii) CTP and (iv) TTP or UTP, wherein each analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, or an analogue thereof, which base does not have a unique label bound thereto, (ii) a deoxyribose, and (iii) a cleavable

moiety bound to the 3'-oxygen of the deoxyribose;

(c) reagents suitable for use in DNA polymerization; and

(d) instructions for use.

This invention further provides the instant kit, wherein the nucleotide analogues of part (a) are 3'-O-allyl-dGTP-allyl-Cy5, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP- allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G. This invention further provides the instant kit, wherein the nucleotide analogues of part (a) are 3'-O-allyl-dGTP-allyl-Bodipy-FL-510, 3'-O-allyl-dCTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G. This invention further provides the instant kit, wherein the nucleotide analogues of part (a) are 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G. This invention further provides the instant kit, wherein the nucleotide analogues of part (b) are 3'-O-allyl-dGTP, 3'-O-allyl-dCTP, 3'-O-allyl-dATP and 3'-O-allyl-dUTP. This invention further provides the instant kit, the reversible terminators in step (c) are 3'-O-2-nitrobenzyl-dGTP, 3'-O-2-nitrobenzyl-dCTP, 3'-O-2-nitrobenzyl-dATP and 3'-O-2-nitrobenzyl-dUTP.

The methods and kits of this invention may be applied, mutatis mutandis, to the sequencing of RNA, or to determining the identity of a ribonucleotide.

Methods for production of cleavably capped and/or cleavably linked nucleotide analogues are disclosed in U.S. Patent No. 6,664,079, which is hereby incorporated by reference. Combinatorial fluorescence energy tags and methods for production thereof are disclosed in U.S. Patent No. 6,627,748, which is hereby incorporated by reference.

In an embodiment, the DNA or nucleic acid is attached /bound to the solid surface by covalent site-specific coupling chemistry compatible with DNA.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

DNA sequencing by synthesis (SBS) on a solid surface during polymerase reaction offers a new paradigm to decipher DNA sequences. Disclosed here is the construction of such a novel DNA sequencing system using molecular engineering approaches. In this approach, four nucleotides (A, C, G, T) are modified as reversible terminators by attaching a cleavable fluorophore to the base and capping the 3′-OH group with a small chemically reversible moiety so that they are still recognized by DNA polymerase as substrates. It is found that an allyl moiety can be used successfully as a linker to tether a fluorophore to 3′-O-allyl-modified nucleotides, forming chemically cleavable fluorescent nucleotide reversible terminators, *3′-O-allyl-dNTPs-allyl-fluorophore*, for application in SBS. The fluorophore and the 3′-O-allyl group on a DNA extension product, which is generated by incorporating *3′-O-allyl-dNTPs-allyl-fluorophore* in a polymerase reaction, are removed simultaneously in 30 seconds by Pd-catalyzed deallylation in aqueous buffer solution. This one-step dual-deallylation reaction thus allows the re-initiation of the polymerase reaction and increases the SBS efficiency. DNA templates consisting of homopolymer regions were accurately sequenced by using this new class of fluorescent nucleotide analogues on a DNA chip and a 4-color fluorescent scanner.

It is known that some modified DNA polymerases are highly tolerable for nucleotides with extensive

modifications with bulky groups such as energy transfer dyes at the 5-position of the pyrimidines (T and C) and 7-position of purines (G and A) (23, 24). The ternary complexes of a rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate have been determined (22) which supports this fact. It was hypothesizrf that if a unique fluorescent dye is linked to the 5-position of the pyrimidines (T and C) and the 7-position of purines (G and A) via a cleavable linker, and a small chemical moiety is used to cap the 3'-OH group, then the resulting nucleotide analogues may be able to incorporate into the growing DNA strand as terminators. Based on this rationale, SBS approach was conceived using cleavable fluorescent nucleotide analogues as reversible terminators to sequence surface-immobilized DNA in 2000 (Fig. 1) (25). In this approach, the nucleotides are modified at two specific locations so that they are still recognized by DNA polymerase as substrates: (i) a different fluorophore with a distinct fluorescent emission is linked to each of the 4 bases through a cleavable linker and (ii) the 3'-OH group is capped by a small chemically reversible moiety. DNA polymerase incorporates only a single nucleotide analogue complementary to the base on a DNA template covalently linked to a surface. After incorporation, the unique fluorescence emission is detected to identify the incorporated nucleotide and the fluorophore is subsequently removed. The 3'-OH group is then chemically regenerated, which allows the next cycle of the polymerase reaction to proceed. Since the large surface on a DNA chip can have a high density of

different DNA templates spotted, each cycle can identify many bases in parallel, allowing the simultaneous sequencing of a large number of DNA molecules. Thee feasibility of performing SBS on a chip using 4 photocleavable fluorescent nucleotide analogues was previously established (26) and it was discovered that an allyl group can be used as a cleavable linker to bridge a fluorophore to a nucleotide (27). The design and synthesis of two photocleavable fluorescent nucleotides as reversible terminators for polymerase reaction has already been reported (28, 29).

Previous research efforts in the present laboratory have firmly established the molecular level strategy to rationally modify the nucleotides by attaching a cleavable fluorescent dye to the base and capping the 3'-OH with a small chemically reversible moiety for SBS. This approach was recently adopted by Genomics Industry to potentially provide a new platform for DNA sequencing (30). Here the design and synthesis of 4 chemically cleavable fluorescent nucleotide analogues as reversible terminators for SBS is disclosed. Each of the nucleotide analogues contains a 3'-O-allyl group and a unique fluorophore with a distinct fluorescence emission at the base through a cleavable allyl linker.

It was first established that these nucleotide analogues are good substrates for DNA polymerase in a solution-phase DNA extension reaction and that the fluorophore and the 3'-O-allyl group can be removed

with high efficiency in aqueous solution. Then SBS was performed using these 4 chemically cleavable fluorescent nucleotide analogues as reversible terminators to identify ~20 continuous bases of a DNA template immobilized on a chip. Accurate DNA sequences were obtained for DNA templates containing homopolymer sequences. The DNA template was immobilized on the surface of the chip that contains a PEG linker with 1,3-dipolar azide-alkyne cycloaddition chemistry. These results indicated that successful cleavable fluorescent nucleotide reversible terminators for 4-color DNA sequencing by synthesis can be designed by attaching a cleavable fluorophore to the base and capping the 3'-OH with a small chemically reversible moiety so that they are still recognized by DNA polymerase as substrates. Further optimization of the approach will lead to even longer sequencing readlengths.

Design and synthesis of chemically cleavable fluorescent nucleotide analogues as reversible terminators for SBS.

To demonstrate the feasibility of carrying out *de novo* DNA sequencing by synthesis on a chip, four chemically cleavable fluorescent nucleotide analogues (3'-O-*allyl*-dCTP-*allyl*-Bodipy-FL-510, 3'-O-*allyl*-dUTP-*allyl*-R6G, 3'-O-*allyl*-dATP-*allyl*-ROX and 3'-O-*allyl*-dGTP-*allyl*-Bodipy-650/Cy5) (Fig. 2) were designed and synthesized as reversible terminators for DNA polymerase reaction. Modified DNA polymerases have been shown to be highly tolerant to nucleotide

modifications with bulky groups at the 5-position of pyrimidines (C and U) and the 7-position of purines (A and G). Thus, each unique fluorophore was attached to the 5 position of C/U and the 7 position of A/G through an allyl carbamate linker. However, due to the close proximity of the 3' position on the sugar ring of a nucleotide to the amino acid residues of the active site of the DNA polymerase, a relatively small allyl moiety was chosen as the 3'-OH reversible capping group. It was found that the fluorophore and the 3'-O-allyl group on a DNA extension product, which is generated by incorporation of the chemically cleavable fluorescent nucleotide analogues, are removed simultaneously in 30 seconds by Pd-catalyzed deallylation in aqueous solution. This one-step dual-deallylation reaction thus allows the re-initiation of the polymerase reaction. The detailed synthesis procedure and characterization of the 4 novel nucleotide analogues in Fig. 2 are described in

Materials and Methods.

In order to verify that these fluorescent nucleotide analogues are incorporated accurately in a base-specific manner in a polymerase reaction, four continuous steps of DNA extension and deallylation were carried out in solution. This allows the isolation of the DNA product at each step for detailed molecular structure characterization by MALDI-TOF mass spectrometry (MS) as shown in Fig. 3. The first extension product 5'--U(allyl-R6G)-3'-O-allyl (**1**) was purified by HPLC and analyzed using MALDI-TOF MS [Fig. 3(A)]. This product was then deallylated using a Pd-

catalyzed deallylation cocktail [1X Thermopol I reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$]. The active Pd catalyst is generated from Na_2PdCl_4 and a ligand $P(PhSO_3Na)_3$ (TPPTS) to mediate the deallylation reaction in DNA compatible aqueous condition to simultaneously cleave both the fluorophore and the 3'-O-allyl group (28). The deallylated product (2) was also analyzed using MALDI-TOF MS [Fig. 3(B)]. As can be seen from Fig. 3(A), the MALDI-TOF MS spectrum consists of a distinct peak at *m/z* 6469 corresponding to the DNA extension product 5'--U(allyl-R6G)-3'-O-allyl (1), which confirms that the nucleotide analogue can be incorporated base specifically among pool of all four (A, C, G, T) by DNA polymerase into a growing DNA strand. Fig. 3(B) shows the deallylation result of the above DNA product. The peak at *m/z* 6469 has completely disappeared while the peak corresponding to the dual deallylated product 5'--U (2) appears as the sole dominant peak at *m/z* 5870, which establishes that the Pd-catalyzed deallylation reaction completely cleaves both the fluorophore and the 3'-O-allyl group with high efficiency without damaging the DNA. The next extension reaction was carried out using this deallylated DNA product with a free 3'-OH group regenerated as a primer along with four allyl modified fluorescent nucleotide mixture to yield an extension product 5'--UG(allyl-Bodipy-650)-3'-O-allyl (3). As described above, the extension product 3 was analyzed by MALDI-TOF MS producing a dominant peak at *m/z* 6984 [Fig. 3(C)], and then deallylated for further MS analysis yielding a single peak at *m/z* 6256 (product 4) [Fig. 3(D)]. The third extension reaction yielding

5'--UGA(allyl-ROX)-3'-O-allyl (5), the fourth extension reaction yielding 5'--UGAC(allyl-Bodipy-FL-510)-3'-O-allyl (7) and their deallylation reactions to yield products **6** and **8** were similarly carried out and analyzed by MALDI-TOF MS as shown in Figs. 3(E), 3(F), 3(G) and 3(H). The chemical structures of the extension and cleavage products for each step are shown in Fig. 4. These results demonstrate that the above-synthesized 4 chemically cleavable fluorescent nucleotide analogues are successfully incorporated with high fidelity into the growing DNA strand in a polymerase reaction, and furthermore, both the fluorophore and the 3'-O-allyl group are efficiently removed by using a Pd-catalyzed deallylation reaction, which makes it feasible to use them for SBS on a chip.

4-Color DNA sequencing with chemically cleavable fluorescent nucleotide analogues as reversible terminators on a DNA chip

The chemically cleavable fluorescent nucleotide analogues were then used in an SBS reaction to identify the sequence of the DNA template immobilized on a solid surface. A site-specific 1,3-dipolar cycloaddition coupling chemistry was used to covalently immobilize the alkyne-labeled self-priming DNA template on the azido-functionalized surface in the presence of a Cu(I) catalyst. The principal advantage offered by the use of a self-priming moiety as compared to using separate primers and templates is that the covalent linkage of the primer to the template in the self-priming moiety prevents any possible dissociation of the primer from the template

during the process of SBS. To prevent non-specific absorption of the unincorporated fluorescent nucleotides on the surface of the chip, a PEG linker is introduced between the DNA templates and the chip surface (Fig. 5). This approach was shown to produce very low background fluorescence after cleavage to remove the fluorophore as demonstrated by the DNA sequencing data described below.

SBS was first performed on a chip-immobilized DNA template that has no homopolymer sequences using the four chemically cleavable fluorescent nucleotide reversible terminators (3'-O-*allyl*-dCTP-*allyl*-Bodipy-FL-510, 3'-O-*allyl*-dUTP-*allyl*-R6G, 3'-O-*allyl*-dATP-*allyl*-ROX and 3'-O-*allyl*-dGTP-*allyl*-Cy5) and the results are shown in Fig. 6. The structure of the self-priming DNA moiety is shown schematically in Fig. 6A, with the first 13 nucleotide sequences immediately after the priming site. The *de novo* sequencing reaction on the chip was initiated by extending the self-priming DNA using a solution containing all four 3'-O-*allyl*-dNTPs-*allyl*-fluorophore, and a 9°N mutant DNA polymerase. In order to negate any lagging fluorescence signal that is caused by previously unextended priming strand, a synchronization step was added to reduce the amount of unextended priming strands after the extension with the fluorescent nucleotides. A synchronization reaction mixture consisting of all four 3'-O-allyl-dNTPs (Fig. 7), which have a higher polymerase incorporation efficiency due to the lack of a fluorophore compared to the bulkier 3'-O-*allyl*-dNTPs-*allyl*-fluorophore, was

used along with the 9°N mutant DNA polymerase to extend any remaining priming strand that has a free 3'-OH group to synchronize the incorporation. The extension by 3'-O-allyl-dNTPs also enhances the enzymatic incorporation of the next nucleotide analogue, because after cleavage to remove the 3'-O-allyl group, the DNA product extended by 3'-O-allyl-dNTPs carry no modification groups. After washing, the extension of the primer by only the complementary fluorescent nucleotide was confirmed by observing a red signal (the emission from Cy5) in a 4-color fluorescent scanner [Fig. 6B (1)]. After detection of the fluorescent signal, the chip surface was immersed in a deallylation cocktail [1X Thermolpol I reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$] and incubated for 5 min at 60 °C to cleave both the fluorophore and 3'-O-allyl group simultaneously. The chip was then immediately immersed in a 3 M Tris-HCl buffer (pH 8.5) and incubated for 5 min at 60 °C to remove the Pd complex. The surface was then washed, and a negligible residual fluorescent signal was detected to confirm cleavage of the fluorophore. This was followed by another extension reaction using 3'-O-*allyl*-dNTPs-*allyl*-fluorophore mixture to incorporate the next fluorescent nucleotide complementary to the subsequent base on the template. The entire process of incorporation, synchronization, detection and cleavage was performed multiple times using the four chemically cleavable fluorescent nucleotide reversible terminators to identify 13 successive bases in the DNA template. The fluorescence image of the chip for each nucleotide addition is shown in Fig. 6B, while a plot

of the fluorescence intensity vs. the progress of sequencing extension (raw 4-color sequencing data) is shown in Fig. 6C. The DNA sequences are unambiguously identified from the 4-color raw fluorescence data without any processing.

Comparison of 4-color SBS with pyrosequencing.

To further verify the advantage of SBS method using the four chemically cleavable fluorescent nucleotide reversible terminators, we carried out similar sequencing reaction as described above on a DNA template which contained two separate homopolymeric regions (stretch of 10 T's and 5 T's) as shown in Fig. 8 (panel A). These sequencing raw data were produced by adding all 4 fluorescent nucleotide reversible terminators together to the DNA template immobilized on the chip followed by synchronization reaction with four 3'-O-allyl-dNTPs, detecting the unique fluorescence emission for sequence determination, then cleaving the fluorophore and the 3'-O-allyl group in one step to continue the sequencing cycles. All 20 bases including the individual base (A, T, C, G), the 10 repeated A's and the 5 repeated A's are clearly identified. The small groups of peaks between the identified bases are fluorescent background from the DNA chip, which does not build up as the cycle continues. Panel B in Fig. 8 shows the pyrosequencing data of the same DNA template containing the homopolymeric sequences. The first 4 individual bases are clearly identified. The two homopolymeric regions (10 A's) and (5 A's) produce two large peaks, but it

is very difficult to identify the exact sequence from the data.

Conclusion

Four novel chemically cleavable fluorescent nucleotide analogues have been synthesized and characterized and have been used to produce a 4-color *de novo* DNA sequencing data on a chip. In doing so, two critical requirements for using SBS method to sequence DNA have been achieved unambiguously. First, a strategy to use a chemically reversible moiety to cap the 3'-OH group of the nucleotide has been successfully implemented so that the nucleotide terminates the polymerase reaction to allow the identification of the incorporated nucleotide. In addition these reversible terminators allow for the addition of all four nucleotides simultaneously in performing SBS. This ultimately reduces the number of cycles needed to complete the sequencing cycle, increases sequencing accuracy due to competition of the 4 nucleotides in the polymerase reaction, and enables accurate determination of homopolymeric regions of DNA template. Second, efficient removal of both the fluorophore and the 3'-OH capping group after the fluorescence signal detection have successfully been carried out which increases the overall efficiency of SBS.

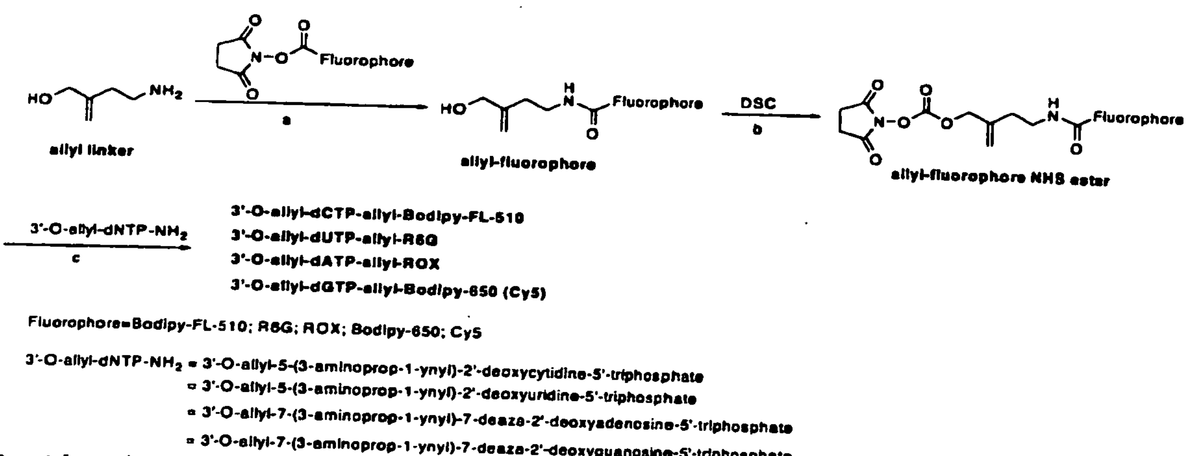
The key factor governing the sequencing readlength of our 4-color SBS approach is the stepwise yield that are determined by the nucleotide incorporation efficiency and the yield of the cleavage of the

fluorophore and the 3'-OH capping group from the DNA extension products. This stepwise yield here is ~ 99% based on measurement of the DNA products in solution phase. The yield on the surface is difficult to measure precisely due to fluctuations in the fluorescence imaging using the current manual fluorescent scanner. The strong fluorescence signal even for the 20^{th} base shown in Fig. 8 indicates that we should be able to extend the readlength even further. In terms of readlength, Sanger sequencing is still the gold standard with readlength of over 800 bp but limited in throughput and cost. The readlength of pyrosequencing is ~100 bp but with high error rate due to difficulty in accurately determining the sequences of homopolymers. The 4-color SBS readlength on a manual fluorescent scanner is currently at ~ 20 bp with high accuracy. This readlength will increase with automation of the extension, cleavage and washing steps. The DNA polymerases and fluorescent labeling used in the automated 4-color Sanger sequencing method have undergone almost two decades of consistent incremental improvements after the basic fluorescent Sanger methods were established (2, 3). Following the same route, it is expected that the basic principle and strategy outlined in our 4-color SBS method will stimulate further improvement of the sequencing by synthesis methodology with engineering of high performance polymerases tailored for the cleavable fluorescent nucleotide terminators and testing alternative linkers and 3'-OH reversible capping moiety. It has been well established that using emulsion PCR on microbeads, millions of different DNA

templates are immobilized on a surface of a chip (11, 16). This high density DNA templates coupled with our 4-color SBS approach will generate a high-throughput (> 20 millions bases/chip) and high-accurate platform for a variety of sequencing and digital gene expression analysis projects.

Materials and Methods

^{1}H NMR spectra were recorded on a Bruker DPX-400 (400 MHz) spectrometer and reported in ppm from a CD_3OD or DMSO-d_6 internal standard (3.31 or 2.50 ppm respectively). Data were reported as follows: (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, ddd = doublet of doublets of doublets; coupling constant(s) in Hz; integration; assignment). Proton decoupled ^{13}C NMR spectra were recorded on a Bruker DPX-400 (100 MHz)

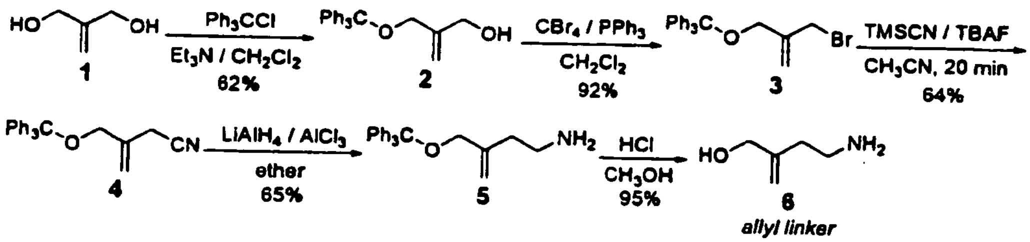


Synthetic scheme to prepare the chemically cleavable fluorescent nucleotides. a, DMF / 1 M $NaHCO_3$ solution; b, *N*, *N'*-disuccinimidyl carbonate (DSC), triethylamine; c, 0.1 M $NaHCO_3$ / Na_2CO_3 aqueous buffer (pH 8.7)

spectrometer and reported in ppm from a CD_3OD, DMSO-d_6 or $CDCl_3$ internal standard (49.0, 39.5 or 77.0 ppm respectively). Proton decoupled ^{31}P NMR spectra were recorded on a Bruker DPX-300 (121.4 MHz) spectrometer and reported in ppm from an 85% H_3PO_4 external standard. High Resolution Mass Spectra (HRMS) were obtained on a JEOL JMS HX 110A mass spectrometer. Compounds 30 and 32 were purchased from Berry & Associates. All Dye NHS esters were purchased from Molecular Probes and GE Healthcare. All other chemicals were purchased from Sigma-Aldrich.

I. Synthesis of 3'-*O*-allyl-dNTPs-allyl-Fluorophore. Chemically cleavable fluorescent nucleotides 3'-*O*-allyl-dCTP-allyl-Bodipy-FL-510, 3'-*O*-allyl-dUTP-allyl-R6G, 3'-*O*-allyl-dATP-allyl-ROX, 3'-*O*-allyl-dGTP-allyl-Bodipy-650 and 3'-*O*-allyl-dGTP-allyl-Cy5 were synthesized according to the above Scheme. A chemically cleavable linker 4-amino-2-methylene-1-butanol (allyl-linker) was reacted with the *N*-hydroxysuccinimide (NHS) ester of the corresponding fluorescent dye to produce an intermediate allyl-fluorophore, which was converted to an allyl-fluorophore NHS ester by reacting with *N,N'*-disuccinimidyl carbonate. The coupling reaction between the different allyl-fluorophore NHS esters and 3'-*O*-allyl-dNTPs-NH_2 produced the four chemically cleavable fluorescent nucleotides.

1. Synthesis of allyl-linker



2-Triphenylmethoxymethyl-2-propen-1-ol (2). To a stirred solution of trityl chloride (4.05 g; 14.3 mmol) and 2-methylenepropane-1,3-diol 1 (1.20 mL; 14.3 mmol) in dry CH_2Cl_2 (20 mL), triethylamine (4.0 mL; 28.5 mmol) was added slowly at room temperature. The mixture was stirred at room temperature for 1 h, and then ethyl acetate (100 mL) and saturated aqueous $NaHCO_3$ (30 mL) were added. The organic phase was washed with saturated aqueous $NaHCO_3$, NaCl, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using ethyl acetate-hexane (1:10~5) as the eluent to afford 2 as a white solid (2.89 g; 62% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.48 (m, 6H, six of ArH), 7.27-7.33 (m, 6H, six of ArH), 7.20-7.27 (m, 3H, three of ArH), 5.26 (s, 1H, one of C=C*H*$_2$), 5.17 (s, 1H, one of C=C*H*$_2$), 4.13 (d, *J* = 6.1 Hz, 2H, C*H*$_2$OH), 3.70 (s, 2H, Ph_3COC*H*$_2$), 1.72 (t, *J* = 6.1 Hz, 1H, CH_2O*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.4, 143.6, 128.3, 127.6, 126.8, 111.6, 87.0, 65.3, 64.5.

1-Bromo-2-triphenylmethoxymethyl-2-propene (3). To a stirred solution of 2 (2.56 g; 7.74 mmol) in CH_2Cl_2 (75 ml), CBr_4 (3.63 g; 10.83 mmol) and triphenylphosphine (2.47 g; 9.31 mmol) were added respectively at 0 °C. The mixture was stirred at room temperature for 40 min.

Ethyl acetate (100 mL) and saturated aqueous $NaHCO_3$ (30 mL) were added at 0°C. The organic phase was washed with saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using CH_2Cl_2-hexane (1:5) as the eluent to afford 3 as white solid (3.02 g; 92% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.48 (m, 6H, six of ArH), 7.27-7.33 (m, 6H, six of ArH), 7.20-7.27 (m, 3H, three of ArH), 5.37 (s, 1H, one of C=CH_2), 5.31 (s, 1H, one of C=CH_2), 4.01 (s, 2H, CH_2Br), 3.75 (s, 2H, Ph_3COCH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.6, 142.6, 128.4, 127.6, 126.9, 115.8, 86.9, 64.2, 33.5.

3-Triphenylmethoxymethyl-3-butenonitrile (4). To a stirred solution of 3 (1.45 g; 3.69 mmol) and in dry CH_3CN (37 mL), trimethylsilyl cyanide (0.49 mL; 3.69 mmol) was added. Then, 1 M tetrabutylammonium fluoride (TBAF) in THF solution (3.69 mL, 3.69 mmol) was added into the above reaction mixture slowly at room temperature. The mixture was stirred for 20 min. After evaporation, the residue was diluted with ethyl acetate (100 mL) and saturated aqueous $NaHCO_3$ (30 mL). The organic phase was washed with saturated aqueous NaCl and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using acetate-hexane (1:10) as the eluent to afford 4 as white solid (1.01 g; 64% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.45 (m, 6H, six of ArH), 7.21-7.34 (m, 9H, nine of ArH), 5.31 (s, 2H, C=CH_2), 3.64 (s, 2H, Ph_3COCH_2), 3.11 (s, 2H, CH_2CN); ^{13}C NMR

(100 MHz, $CDCl_3$) δ 143.3, 135.5, 128.2, 127.7, 126.9, 116.8, 114.7, 87.0, 65.7, 21.9.

3-Triphenylmethoxymethyl-3-buten-1-amine (5). To a stirred solution of $LiAlH_4$ (119 mg; 2.98 mmol) in dry ether (5 mL), $AlCl_3$ (400 mg; 2.94 mmol) was added slowly at 0 °C and the mixture was stirred for 15 min. The mixture of 4 (829 mg; 2.44 mmol) in dry ether (9 mL) was added and then continued to stir at 0 °C for another 3h. Afterwards, 10% aqueous NaOH (10 mL) was added to quench the reaction. The organic phase was washed with saturated aqueous $NaHCO_3$, NaCl, and dried over anhydrous K_2CO_3. After evaporation, the residue was further purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20~5) as the eluent to afford 5 as colorless oil (545 mg; 65% yield): ^1H NMR (400 MHz, $CDCl_3$) δ 7.41-7.48 (m, 6H, six of ArH), 7.26-7.33 (m, 6H, six of ArH), 7.19-7.26 (m, 3H, three of ArH), 5.33 (s, 1H, one of C=CH_2), 4.96 (s, 1H, one of C=CH_2), 3.53 (s, 2H, Ph_3COCH_2), 2.70 (m, 2H, $CH_2CH_2NH_2$), 2.18 (t, J = 6.7 Hz, 2H, $CH_2CH_2NH_2$), 2.06 (br s, 2H, NH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.6, 143.4, 128.1, 127.4, 126.5, 111.3, 86.5, 66.1, 39.8, 37.4; HRMS (FAB+) calcd for $C_{24}H_{26}ON$ ($M+H^+$): 344.2014, found: 344.2017.

4-Amino-2-methylene-1-butanol (6). To a stirred solution of 5 (540 mg; 1.57 mmol) in CH_3OH (11 mL), HCl (2M solution in ether; 5.5 mL) was added at room temperature and the mixture was stirred for 1 h. Then 7M ammonia in CH_3OH solution (2.7 mL) was added into the above mixture at room temperature and continued to

stir for another 10 min. After filtration, the solid was washed with CH_3OH and combined with the filtrate. After evaporation, the crude product was further purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:4) as the eluent to afford 6 as colorless oil (151 mg; 95% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 5.19 (s, 1H, one of C=CH_2), 5.01 (s, 1H, one of C=CH_2), 4.06 (s, 2H, CH_2OH), 3.10 (t, J = 7.5 Hz, 2H, CH_2C$H_2$$NH_2$), 2.46 (t, J = 7.5 Hz, 2H, C$H_2$$CH_2NH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 145.3, 113.7, 65.5, 39.5, 32.0; MS (FAB+) calcd for $C_5H_{12}ON$ (M+H$^+$): 102.09, found: 102.12.

2. Synthesis of Allyl-Fluorophore

A general procedure for the synthesis of Allyl-Fluorophore is as follows. To a stirred solution of 6 (3.5 mg; 34.6 μmol) in DMF (450 μL), aqueous $NaHCO_3$ (1M solution; 100 μL) was added at room temperature. The mixture was stirred for 5 min. Dye NHS (*N*-hydroxysuccinimide) ester (5 mg) in DMF (450 μL) was added and then the mixture was stirred at room temperature for 6 h. The crude product was further purified by a preparative TLC plate using CH_3OH-CH_2Cl_2 as the eluent to afford Allyl-Fluorophore.

Allyl-Bodipy-FL-510 (7). ^{1}H NMR (400 MHz, CD_3OD) δ 7.42 (s, 1H), 7.00 (d, J = 4.0 Hz, 1H), 6.32 (d, J = 4.0 Hz, 1H), 6.21 (s, 1H), 5.06 (s, 1H, one of C=CH_2), 4.87 (s, 1H, one of C=CH_2, partly superimposed by solvent signal), 4.01 (s, 2H, CH_2OH), 3.33 (t, J = 7.5 Hz, 2H, partly superimposed by solvent signal), 3.21 (t, J = 7.7 Hz, 2H), 2.59 (t, J = 7.7 Hz, 2H), 2.51 (s, 3H,

one of ArCH_3), 2.28 (s, 3H), 2.26 (t, J = 7.1 Hz, 2H); HRMS (FAB+) calcd for $C_{19}H_{24}O_2N_3F_2B$ (M^+): 375.1933, found: 375.1957.

Allyl-R6G (8). ^{1}H NMR (400 MHz, CD_3OD) δ 8.12 (d, J = 8.1 Hz, 1H), 8.05 (dd, J = 1.8, 8.1 Hz, 1H), 7.66 (d, J = 1.6 Hz, 1H), 7.02 (s, 2H), 6.88 (s, 2H), 5.08 (s, 1H, one of C=CH_2), 4.92 (s, 1H, one of C=CH_2), 4.06 (s, 2H, CH_2OH), 3.48-3.56 (m, 6H), 2.40 (t, J = 7.2 Hz, 2H), 2.13 (s, 6H), 1.36 (t, J = 7.2 Hz, 6H); HRMS (FAB+) calcd for $C_{32}H_{36}O_5N_3$ ($M+H^+$): 542.2655, found: 542.2648.

Allyl-ROX (9). ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (d, J = 8.1 Hz, 1H), 7.98 (dd, J = 1.6, 8.1 Hz, 1H), 7.60 (d, J = 1.4 Hz, 1H), 6.75 (s, 2H), 5.08 (s, 1H, one of C=CH_2), 4.91 (s, 1H, one of C=CH_2), 4.05 (s, 2H, CH_2OH), 3.45-3.57 (m, 10H), 3.03-3.10 (m, 4H), 2.64-2.73 (m, 4H), 2.38 (t, J = 7.1 Hz, 2H), 2.04-2.15 (m, 4H), 1.89-1.99 (m, 4H); HRMS (FAB+) calcd for $C_{38}H_{40}O_5N_3$ ($M+H^+$): 618.2968, found: 618.2961.

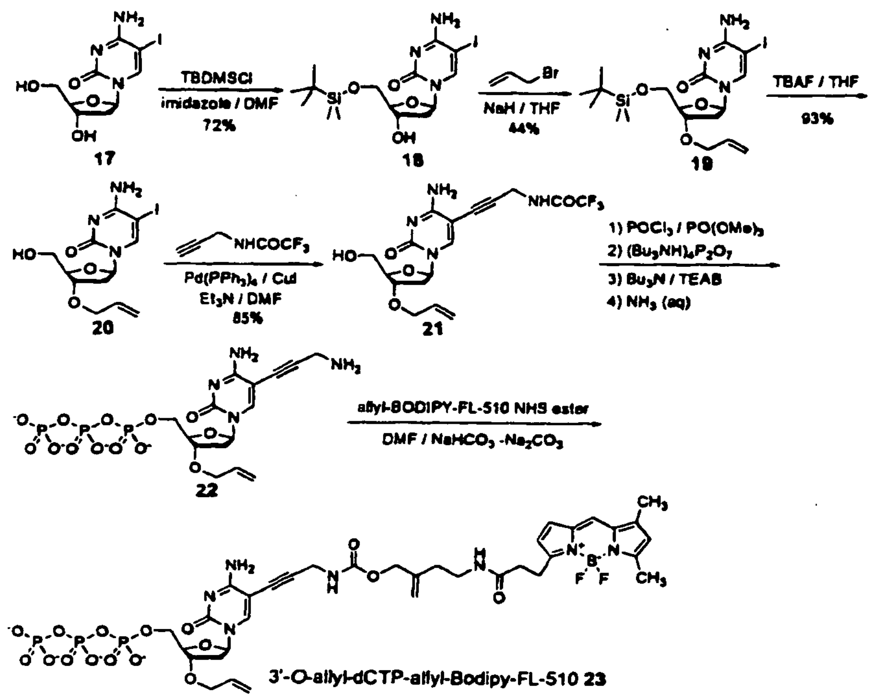
Allyl-Bodipy-650 (10). ^{1}H NMR (400 MHz, CD_3OD) δ 8.12 (dd, J = 0.9, 3.8 Hz, 1H), 7.63 (m, 3H), 7.54 (d, J = 6.4 Hz, 2H), 7.35 (s, 1H), 7.18-7.22 (m, 2H), 7.12 (m, 2H), 7.06 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 4.2 Hz, 1H), 5.06 (s, 1H, one of C=CH_2), 4.86 (s, 1H, one of C=CH_2),4.56 (s, 2H), 4.00 (s, 2H, CH_2OH), 3.28 (m, 4H), 2.23 (t, J = 7.1 Hz, 2H), 2.14 (t, J = 7.5 Hz, 2H), 1.49-1.62 (m, 4H), 1.25-1.34 (m, 2H); HRMS (FAB+) calcd for $C_{34}H_{37}O_4N_4F_2SB$ (M^+): 646.2603, found: 646.2620.

Allyl-Cy5 (11). ^{1}H NMR (400 MHz, CD_3OD) δ 7.75-7.86 (m, 2H), 7.43-7.62 (m, 3H), 6.65 (m, 1H), 6.25-6.53 (m, 5H), 5.06 (s, 1H, one of $C{=}CH_2$), 4.86 (s, 1H, one of $C{=}CH_2$), 4.56 (s, 2H), 4.00 (s, 2H, CH_2OH), 3.28 (m, 6H), 2.03-2.40 (m, 4H), 1.55 (t, J = 7.2 Hz, 3H), 1.31 (s, 6H), 1.26 (s, 6H), 1.25-1.64 (m, 6H); HRMS (FAB+) calcd for $C_{38}H_{48}O_8N_3S_2$ (M^+): 738.2888, found: 738.2867.

3. Synthesis of Allyl-Fluorophore NHS ester

A general procedure for the synthesis of Allyl-Dye NHS ester is as follows. To a stirred solution of Allyl-Fluorophore (4 mg) in dry DMF (350 μL), DSC (8.0 mg; 31.2 μmol) and triethylamine (5.0 μL; 35.4 μmol) were added respectively. The reaction mixture was stirred at room temperature for 10 h. After evaporation, the crude product was further purified by flash column chromatography using CH_3OH-CH_2Cl_2 as the eluent to afford Allyl-Fluorophore NHS ester, which was used directly for the next step.

4. Synthesis of 3'-O-allyl-dCTP-allyl-Bodipy-FL-510 (23)



5'-*O*-(*tert*-Butyldimethylsilyl)-5-iodo-2'-deoxycytidine (18). To a stirred mixture of 17 (1.00 g; 2.83 mmol) and imidazole (462 mg; 6.79 mmol) in anhydrous DMF (14.0 mL), *tert*-butyldimethylsilyl chloride (TBDMSCl) (510 mg; 3.28 mmol) was added. The reaction mixture was stirred at room temperature for 20 h. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20) as the eluent to afford 18 as white solid (1.18 g; 89% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.18 (s, 1H, 6-H), 6.17 (dd, J = 5.8, 7.5 Hz, 1H, 1'-H), 4.34 (m, 1H, 3'-H), 4.04 (m, 1H, 4'-H), 3.93 (dd, J = 2.5, 11.6 Hz, 1H, one of 5'-H), 3.84 (dd, J = 2.9, 11.6 Hz, 1H, one of 5'-H), 2.41-2.48 (ddd, J = 2.5, 5.8, 13.5 Hz, 1H, one of 2'-H), 2.01-2.08 (ddd, J = 5.9, 7.6, 13.5 Hz, 1H, one of 2'-H), 0.95 (s, 9H, $C(CH_3)_3$), 0.17 (s, 3H, one of $SiCH_3$),

0.16 (s, 3H, one of SiCH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 165.5, 156.8, 147.8, 89.4, 88.3, 72.8, 64.6, 57.1, 43.1, 26.7, 19.4, -4.8, -4.9; HRMS (FAB+) calcd for $C_{15}H_{27}O_4N_3SiI$ (M+H$^+$): 468.0816, found: 468.0835.

3'-*O*-Allyl-5'-*O*-(*tert*-butyldimethylsilyl)-5-iodo-2'-deoxycytidine (19). To a stirred solution of 18 (1.18 g; 2.52 mmol) in anhydrous THF (43 mL), 95% NaH powder (128 mg; 5.07mmol) was added. The suspension mixture was stirred at room temperature for 45 min. Allyl bromide (240 µL, 2.79 mmol) was then added at 0 °C and the reaction was stirred at room temperature for another 14 h with exclusion of moisture. Saturated aqueous $NaHCO_3$ (10 mL) was added at 0 °C and the reaction mixture was stirred for 10 min. After evaporation, the residue was dissolved in ethyl acetate (150 mL). The solution was then washed with saturated aqueous $NaHCO_3$ and NaCl, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using ethyl acetate as the eluent to afford 19 as white solid (601 mg; 47% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.15 (s, 1H, 6-H), 6.12 (dd, *J* = 5.6, 8.0 Hz, 1H, 1'-H), 4.17 (m, 1H, 4'-H), 4.14 (m, 1H, 3'-H), 3.98-4.10 (m, 2H, $CH_2CH{=}CH_2$), 3.93 (dd, *J* = 2.8, 11.5 Hz, 1H, one of 5'-H), 3.83 (dd, *J* = 2.8, 11.5 Hz, 1H, one of 5'-H), 2.53-2.60 (ddd, *J* = 1.7, 5.6, 13.6 Hz, 1H, one of 2'-H), 1.94-2.02 (ddd, *J* = 5.9, 8.0, 13.6 Hz, 1H, one of 2'-H), 0.94 (s, 9H, C(CH_3)$_3$), 0.17 (s, 3H, one of SiCH_3), 0.16 (s, 3H, one of SiCH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 165.4, 156.7, 147.7, 135.5, 117.2, 88.2, 87.0,

80.4, 70.9, 64.8, 57.3, 40.1, 26.7, 19.4, -4.7, -4.9; HRMS (FAB+) calcd for $C_{18}H_{31}O_4N_3SiI$ $(M+H^+)$: 508.1129, found: 508.1123.

3'-*O*-Allyl-5-iodo-2'-deoxycytidine (20). To a stirred solution of 19 (601 mg; 1.18 mmol) in anhydrous THF (28 mL), 1 M TBAF in THF solution (1.31 mL; 1.31 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. After evaporation, the residue was dissolved in ethyl acetate (100 mL). The solution was then washed with saturated aqueous NaCl and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:10) as the eluent to afford 20 as white crystals (329 mg; 71% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.47 (s, 1H, 6-H), 6.15 (dd, J = 6.2, 6.7 Hz, 1H, 1'-H), 5.87-5.98 (m, 1H, $CH_2CH{=}CH_2$), 5.26-5.33 (dm, J = 17.2 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.14-5.19 (dm, J = 10.5 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.18 (m, 1H, 3'-H), 4.08 (m, 1H, 4'-H), 3.98-4.10 (m, 2H, $CH_2CH{=}CH_2$), 3.82 (dd, J = 3.2, 13.0 Hz, 1H, one of 5'-H), 3.72 (dd, J = 3.3, 13.0 Hz, 1H, one of 5'-H), 2.44-2.51 (ddd, J = 3.2, 6.0, 13.6 Hz, 1H, one of 2'-H), 2.07-2.15 (m, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 165.4, 156.9, 148.8, 135.6, 117.0, 87.9, 86.9, 79.6, 71.2, 62.7, 57.2, 39.7; HRMS (FAB+) calcd for $C_{12}H_{17}O_4N_3I$ $(M+H^+)$: 394.0264, found: 394.0274.

3'-*O*-Allyl-5-{3-[(trifluoroacetyl)amino]prop-1-ynyl}-2'-deoxycytidine (21). To a stirred solution of 20 (329 mg; 0.84 mmol) in anhydrous DMF (3.7 mL),

tetrakis(triphenylphosphine)palladium(0) (97 mg; 0.084 mmol) and CuI (35 mg; 0.18 mmol) were added. The reaction mixture was stirred at room temperature for 10 min. Then *N*-propargyltrifluoroacetamide (379 mg; 2.51 mmol) and Et_3N (233 μL; 1.68 mmol) were added into the above reaction mixture. The reaction was stirred at room temperature for 1.5 h with exclusion of air and light. After evaporation, the residue was dissolved in ethyl acetate (100 mL). The mixture was washed with saturated aqueous $NaHCO_3$, NaCl, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (0-1:10) as the eluent to afford 21 as yellow crystals (290 mg; 83% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.31 (s, 1H, 6-H), 6.17 (dd, J = 6.0, 7.3 Hz, 1H, 1′-H), 5.87-5.97 (m, 1H, $CH_2CH{=}CH_2$), 5.26-5.33 (dm, J = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.15-5.19 (dm, J = 10.4 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.31 (s, 2H, $C{\equiv}CCH_2$), 4.17 (m, 1H, 3′-H), 4.09 (m, 1H, 4′-H), 3.98-4.10 (m, 2H, $CH_2CH{=}CH_2$), 3.80 (dd, J = 3.4, 12.0 Hz, 1H, one of 5′-H), 3.72 (dd, J = 3.6, 12.0 Hz, 1H, one of 5′-H), 2.46-2.53 (ddd, J = 2.9, 5.3, 13.6 Hz, 1H, one of 2′-H), 2.04-2.12 (m, 1H, one of 2′-H); ^{13}C NMR (100 MHz, CD_3OD) δ 166.0, 158.4 (q, J = 38 Hz, $COCF_3$), 156.3, 145.8, 135.6, 117.1 (q, J = 284 Hz, $COCF_3$), 117.0, 91.9, 90.7, 88.0, 87.0, 79.8, 75.5, 71.2, 62.8, 39.6, 31.0; HRMS (FAB+) calcd for $C_{17}H_{20}O_5N_4F_3$ $(M+H^+)$: 417.1386, found: 417.1377.

3′-*O*-Allyl-5-(3-aminoprop-1-ynyl)-2′-deoxycytidine-5′-triphosphate (22). 21 (133 mg; 0.319 mmol) and proton

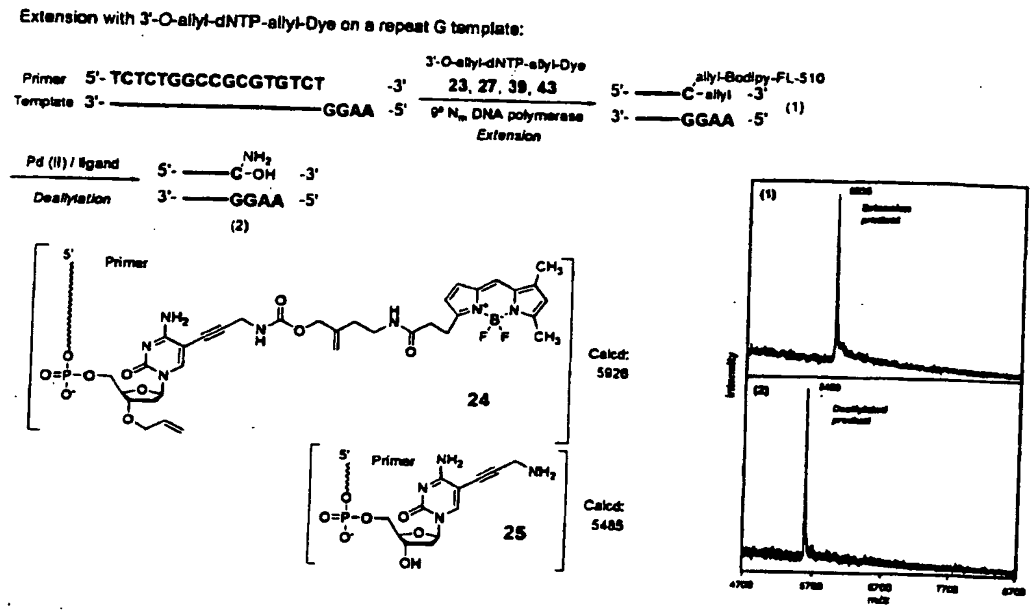
sponge (83.6 mg; 0.386 mmol) were dried in a vacuum desiccator over P_2O_5 overnight before dissolving in trimethylphosphate (0.60 mL). Freshly distilled $POCl_3$ (36 μL; 0.383 mmol) was added dropwise at 0 °C and the mixture was stirred for 3 h. Then the solution of tributylammonium pyrophosphate (607 mg) and tributylamine (0.61 mL; 2.56 mmol) in anhydrous DMF (2.56 mL) was well vortexed and added in one portion at room temperature and the reaction mixture was stirred for 30 min. After that triethylammonium bicarbonate solution (TEAB) (0.1 M; 16 mL) was added and the mixture was stirred for 1 h. Then aqueous ammonia (28%; 16 mL) was added and the reaction mixture was stirred for 12 h. After most liquid was removed under vacuum, the residue was redissolved in water (2 mL) and filtered. The aqueous solution was purified by DEAE Sephadex A25 ion exchange column using gradient aqueous TEAB solution (from 0.1 M to 1.0 M) as eluent to afford 22 as colorless syrup after evaporation: ^{1}H NMR (300 MHz, D_2O) δ 8.43 (s, 1H, 6-H), 6.21 (t, J = 6.7 Hz, 1H, 1'-H), 5.85-6.00 (m, 1H, $CH_2CH{=}CH_2$), 5.28-5.38 (dm, J = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.19-5.27 (dm, J = 10.4 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.22-4.41 (m, 3H, 3'-H and $C{\equiv}CCH_2$), 4.05-4.18 (m, 3H, 4'-H and $CH_2CH{=}CH_2$), 3.94-4.01 (m, 2H, 5'-H), 2.47-2.59 (m, 1H, one of 2'-H), 2.20-2.32 (m, 1H, one of 2'-H); ^{31}P NMR (121.4 MHz, D_2O) δ -7.1 (d, J = 19.8 Hz, 1P, γ-P), -11.1 (d, J = 19.1 Hz, 1P, α-P), -21.9 (t, J = 19.5 Hz, 1P, β-P).

3'-*O*-Allyl-dCTP-allyl-Bodipy-FL-510 (23). To a stirred solution of allyl-Bodipy-FL-510 NHS ester 12 in DMF (300 μL), 3'-*O*-allyl-dCTP-NH_2 22 in 1M $NaHCO_3$-Na_2CO_3 buffer (300 μL; pH 8.7) was added. The reaction mixture was stirred at room temperature for 7 h and the crude product was purified by a preparative TLC plate using CH_3OH-CH_2Cl_2 (1:1) as the eluent. The crude product was further purified by reverse-phase HPLC using a 150x4.6 mm C18 column to afford compound 23 (retention time = 35 min). Mobile phase: A, 4.4 mM Et_3N / 98.3 mM 1,1,1,3,3,3-hexafluoro-2-propanol in water (pH = 8.1); B, methanol. Elution was performed from 100% A isocratic over 10 min followed by a linear gradient of 0-50% B for 20 min and then 50% B isocratic over 20 min. The product was characterized by the following single base extension reaction to generate DNA extension product 24 and characterized by MALDI-TOF MS.

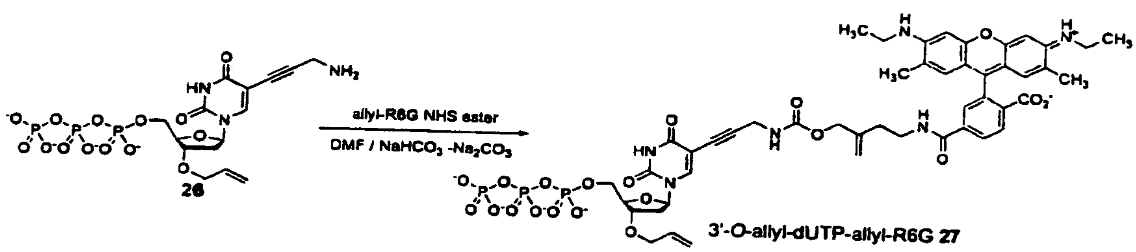
A general procedure of primer extension using 3'-*O*-allyl-dNTPs-allyl-Fluorophore. The polymerase extension reaction mixture consisted of 50 pmol of primer (5'-GTTGATGTACACATTGTCAA-3'), 80 pmol of 100-mer template (5'-TACCCGGAGGCCAAGTACGGCGGGTACGTCCTTGACAATGTGTACATCAACATCACCTACCACCATGTCAGTCTCGGTTGGATCCTCTATTGTGTCCGGG-3'), 120 pmol of 3'-*O*-allyl-dNTP-allyl-Fluorophore, 1X Thermopol II reaction buffer [20 mM Tris-HCl/ 10 mM $(NH_4)_2SO_4$/10 mM KCl/ 2 mM $MnCl_2$/0.1 % Triton X-100, pH 8.8, New England Biolabs], and 6 units of 9° N Polymerase (exo-)A485L/Y409V in a total volume of 20 μL. The reaction consisted of 20 cycles at 94 °C for

20 sec, 46 °C for 40 sec, and 60 °C for 90 sec. After the reaction, a small portion of the DNA extension product was desalted using a ZipTip and analyzed by MALDI-TOF MS, which showed a dominant peak at m/z 5935 corresponding to the DNA extension product generated by incorporating 23. The rest of the product was subjected to the following deallylation.

General one-pot dual-deallylation procedure of DNA extension products. The DNA product from above (20 pmol) was added to a mixture of degassed 1X Thermopol I reaction buffer (20 mM Tris-HCl/10 mM $(NH_4)_2SO_4$/10 mM KCl/2 mM $MgSO_4$/0.1 % Triton X-100, pH 8.8, 1 µL), Na_2PdCl_4 in degassed H_2O (7 µL, 23 nmol) and $P(PhSO_3Na)_3$ in degassed H_2O (10 µL, 176 nmol) to perform an one-pot dual-deallylation reaction. The reaction mixture was then placed in a heating block and incubated at 70 °C for 30 seconds to yield quantitatively deallylated DNA product and characterized by MALDI-TOF MS as a single peak.



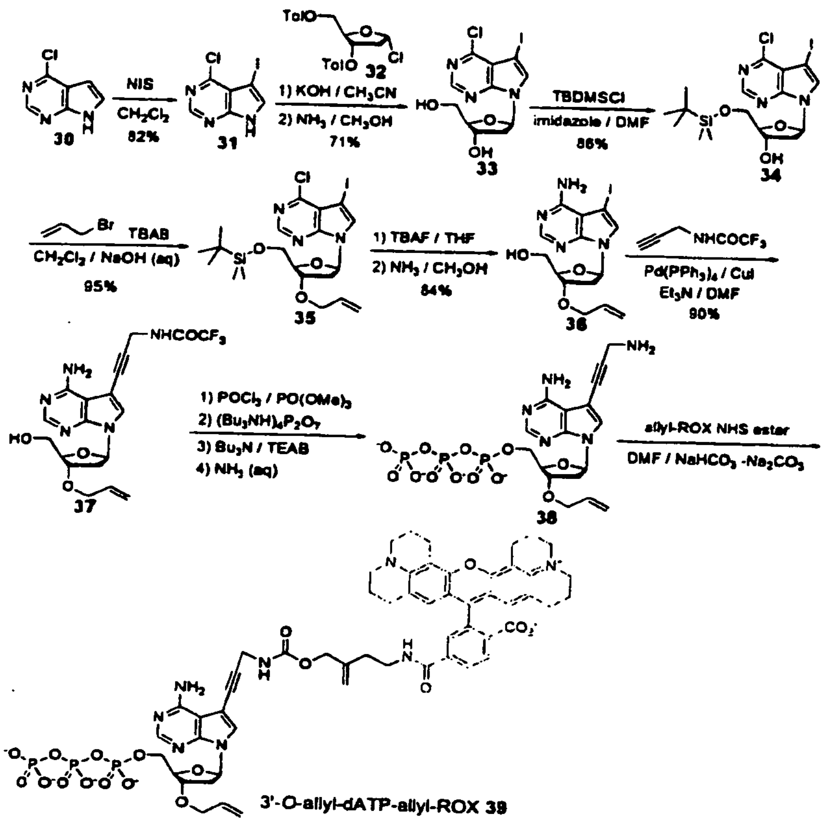
5. Synthesis of 3′-O-allyl-dUTP-allyl-R6G (27)



Synthesis of 3′-O-allyl-dUTP-NH_2 26 was performed according to the procedures in reference (28).

3′-O-Allyl-dUTP-allyl-R6G (27). The procedure was similar to the synthesis of 23. The product was characterized by the single base extension reaction and MALDI-TOF MS in Fig. 9.

6. Synthesis of 3′-*O*-allyl-dATP-allyl-ROX (39)



6-Chloro-7-iodo-7-deazapurine (31). To a vigorously stirred solution of 30 (1.0 g; 6.51 mmol) in CH_2Cl_2 (55 mL), *N*-iodosuccimide (1.70 g; 7.18 mmol) was added. The suspension mixture was stirred at room temperature for 1 h, during which more precipitate was observed. The precipitate was filtered and then recrystallized in hot methanol to afford 31 (1.49 g; 82% yield): ^{1}H NMR (400 MHz, DMSO-d6) δ 12.96 (br s, 1H, N*H*), 8.59 (s, 1H, 2-H), 7.94 (s, 1H, 8-H); ^{13}C NMR (100 MHz, DMSO-d6) δ 151.2, 150.4, 150.2, 133.6, 115.5, 51.7; HRMS (FAB+) calcd for $C_6H_4N_3ClI$ $(M+H^+)$: 279.9139, found: 279.9141.

6-Chloro-9-(β-D-2'-deoxyribofuranosyl)-7-iodo-7-deazapurine (33). To a stirred solution of 31 (707 mg; 2.53 mmol) in CH_3CN (43 mL), KOH powder (355 mg; 6.34 mmol) and tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1) (52 μL, 0.165 mmol) were added. The mixture was stirred at room temperature for 10 min and then 3,5-di-O-(p-toluyl)-2-deoxy-D-ribofuranosyl chloride 32 (1.18 g; 2.73 mmol) was added. The reaction mixture was stirred vigorously at room temperature for 1 h, and the insoluble portion was filtered and washed with hot acetone. The combined solution was evaporated and dissolved in 7M ammonia in methanol solution (86 mL). The mixture was stirred at room temperature for 24 h. After evaporation, the crude product was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (0~1:20) as the eluent to afford 33 as white solid (711 mg; 71% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.57 (s, 1H, 2-H), 8.08 (s, 1H, 8-H), 6.72 (dd, J = 6.3, 7.5 Hz, 1H, 1'-H), 4.53 (m, 1H, 3'-H), 4.00 (m, 1H, 4'-H), 3.80 (dd, J = 3.6, 12.0 Hz, 1H, one of 5'-H), 3.74 (dd, J = 3.6, 12.0 Hz, 1H, one of 5'-H), 2.56-2.64 (ddd, J = 6.1, 7.5, 13.5 Hz, 1H, one of 2'-H), 2.36-2.43 (ddd, J = 3.3, 6.2, 13.5 Hz, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 152.9, 151.7, 151.3, 134.7, 118.5, 89.0, 85.7, 72.6, 63.2, 52.6, 41.7; HRMS (FAB+) calcd for $C_{11}H_{12}O_3N_3ClI$ ($M+H^+$): 395.9612, found: 395.9607.

9-[β-D-5'-O-(*tert*-Butyldimethylsilyl)-2'-deoxyribofuranosyl]-6-chloro-7-iodo7-deazapurine (34). The procedure was similar to the synthesis of 18, and

the crude product was purified by flash column chromatography using ethyl acetate-hexane (1:3~2) as the eluent to afford 34 as white solid (597 mg; 65% yield) and 33 (213 mg; 30% yield). The above procedure was repeated with the recovered 33 to achieve a 86% overall yield of 34: ^{1}H NMR (400 MHz, CD_3OD) δ 8.56 (s, 1H, 2-H), 7.99 (s, 1H, 8-H), 6.73 ('t', J = 6.7 Hz, 1H, 1'-H), 4.52 (m, 1H, 3'-H), 4.02 (m, 1H, 4'-H), 3.92 (dd, J = 3.0, 11.4 Hz, 1H, one of 5'-H), 3.86 (dd, J = 3.1, 11.4 Hz, 1H, one of 5'-H), 2.47-2.55 (ddd, J = 5.8, 7.1, 13.4 Hz, 1H, one of 2'-H), 2.40-2.47 (ddd, J = 3.6, 6.3, 13.4 Hz, 1H, one of 2'-H), 0.94 (s, 9H, $C(CH_3)_3$), 0.14 (s, 3H, one of $SiCH_3$), 0.13 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 152.8, 151.5, 151.3, 133.8, 118.2, 88.9, 85.4, 72.5, 64.6, 52.6, 42.4, 26.7, 19.5, -4.9, -5.0; HRMS (FAB+) calcd for $C_{17}H_{26}O_3N_3ClSiI$ ($M+H^+$): 510.0477, found: 510.0487.

9-[*β*-D-3'-*O*-Allyl-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxyribofuranosyl]-6-chloro-7-iodo-7-deazapurine (35). To a stirred solution of 34 (789 mg; 1.55 mmol) in CH_2Cl_2 (48 mL), tetrabutylammonium bromide (TBAB) (255 mg; 0.77 mmol), allyl bromide (0.69 mL, 7.74 mmol) and 40% aqueous NaOH solution (24 mL) were added respectively. The reaction mixture was stirred at room temperature for 1h. Ethyl acetate (150 mL) was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (2x50 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$, NaCl, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash

column chromatography using ethyl acetate-hexane (1:6) as the eluent to afford 35 as yellow oil (809 mg; 95% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.52 (s, 1H, 2-H), 7.94 (s, 1H, 8-H), 6.64 (dd, J = 6.1, 7.6 Hz, 1H, 1'-H), 5.88-5.99 (m, 1H, $CH_2CH{=}CH_2$), 5.28-5.34 (dm, J = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.16-5.21 (dm, J = 10.4 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.28 (m, 1H, 3'-H), 4.13 (m, 1H, 4'-H), 4.01-4.11 (m, 2H, $CH_2CH{=}CH_2$), 3.88 (dd, J = 3.6, 11.2 Hz, 1H, one of 5'-H), 3.80 (dd, J = 3.1, 11.3 Hz, 1H, one of 5'-H), 2.51-2.57 (ddd, J = 2.7, 6.0, 13.5 Hz, 1H, one of 2'-H), 2.42-2.50 (ddd, J = 5.7, 7.7, 13.5 Hz, 1H, one of 2'-H), 0.93 (s, 9H, $C(CH_3)_3$), 0.13 (s, 3H, one of $SiCH_3$), 0.12 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 152.8, 151.4, 151.3, 135.5, 133.6, 118.2, 117.2, 86.5, 85.6, 80.2, 71.0, 64.8, 52.8, 39.7, 26.7, 19.4, -4.8, -5.0; HRMS (FAB+) calcd for $C_{20}H_{30}O_3N_3ClSiI$ $(M+H^+)$: 550.0790, found: 550.0773.

3'-*O*-Allyl-7-deaza-7-iodo-2'-deoxyadenosine (36). To a stirred solution of 35 (809 mg; 1.47 mmol) in anhydrous THF (34 mL), 1 M TBAF in THF solution (1.62 mL; 1.62 mmol) was added and the reaction was stirred at room temperature for 1 h. After evaporation, the residue was dissolved in 7M ammonia in methanol solution (24 mL). The solution was stirred in an autoclave at 115-120 °C for 15 h. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20) as the eluent to afford 36 as white solid (514 mg; 84% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (s, 1H, 2-H), 7.56 (s, 1H, 8-H),

6.45 (dd, *J* = 5.8, 8.6 Hz, 1H, 1'-H), 5.90-6.00 (m, 1H, $CH_2CH{=}CH_2$), 5.29-5.35 (dm, *J* = 17.2 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.16-5.21 (dm, *J* = 10.5 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.28 (m, 1H, 3'-H), 4.12 (m, 1H, 4'-H), 4.02-4.12 (m, 2H, $CH_2CH{=}CH_2$), 3.78 (dd, *J* = 3.7, 12.1 Hz, 1H, one of 5'-H), 3.70 (dd, *J* = 3.6, 12.1 Hz, 1H, one of 5'-H), 2.53-2.61 (ddd, *J* = 5.8, 8.6, 13.6 Hz, 1H, one of 2'-H), 2.41-2.47 (ddd, *J* = 2.0, 5.8, 13.5 Hz, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.5, 152.3, 150.3, 135.7, 128.8, 117.0, 105.3, 86.8, 86.4, 80.7, 71.0, 63.7, 51.3, 38.8; HRMS (FAB+) calcd for $C_{14}H_{18}O_3N_4I$ $(M+H^+)$: 417.0424, found: 417.0438.

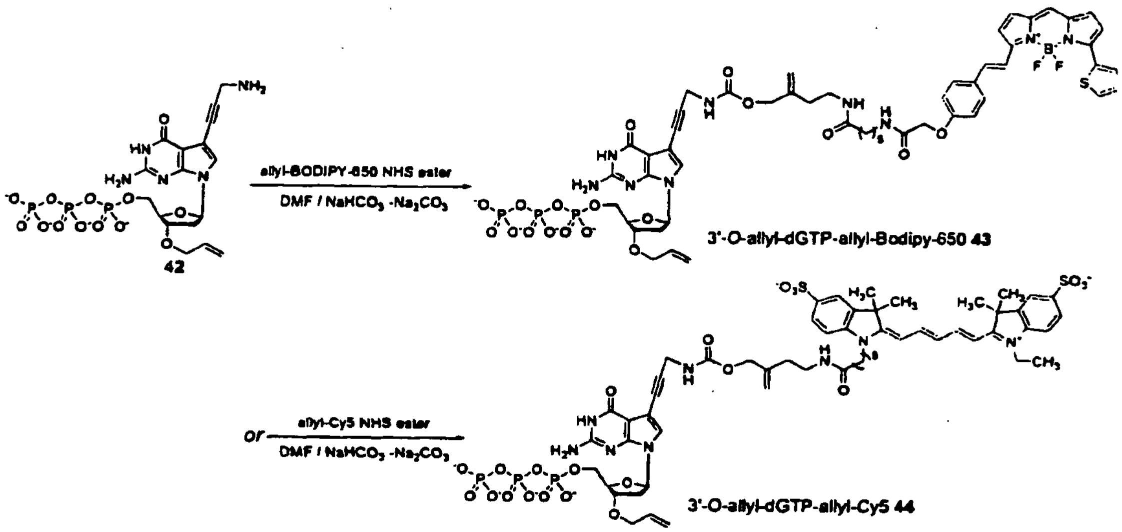
3'-*O*-Allyl-7-deaza-7-{3-[(trifluoroacetyl)amino]prop-1-ynyl}-2'-deoxyadenosine (37). The procedure was similar to the synthesis of 21, and the crude product was purified by flash column chromatography using ethyl acetate-hexane (1:1~0) as the eluent to afford 37 as yellow solid (486 mg; 90% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.08 (s, 1H, 2-H), 7.60 (s, 1H, 8-H), 6.41 (dd, *J* = 5.8, 8.6 Hz, 1H, 1'-H), 5.89-6.00 (m, 1H, $CH_2CH{=}CH_2$), 5.29-5.35 (dm, *J* = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.16-5.21 (dm, *J* = 10.4 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.31 (s, 2H, $C{\equiv}CCH_2$), 4.29 (m, 1H, 3'-H), 4.13 (m, 1H, 4'-H), 4.01-4.11 (m, 2H, $CH_2CH{=}CH_2$), 3.79 (dd, *J* = 3.6, 12.1 Hz, 1H, one of 5'-H), 3.71 (dd, *J* = 3.5, 12.1 Hz, 1H, one of 5'-H), 2.54-2.62 (ddd, *J* = 5.8, 8.6, 13.6 Hz, 1H, one of 2'-H), 2.42-2.48 (ddd, *J* = 1.9, 5.8, 13.6 Hz, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.8, 158.6 (q, *J* = 38 Hz, $COCF_3$), 152.9, 149.6, 135.6, 128.1, 117.1 (q, *J* = 284 Hz, $COCF_3$),

117.0, 104.5, 96.3, 87.3, 86.9, 86.8, 80.7, 77.0, 71.0, 63.8, 38.7, 31.1; HRMS (FAB+) calcd for $C_{19}H_{21}O_4N_5F_3$ ($M+H^+$): 440.1546, found: 440.1544.

3'-*O*-Allyl-7-(3-aminoprop-1-ynyl)-7-deaza-2'-deoxyadenosine-5'-triphosphate (38). The procedure was similar to the synthesis of 22 to yield 38 as colorless syrup: ^1H NMR (300 MHz, D_2O) δ 8.02 (s, 1H, 2-H), 7.89 (s, 1H, 8-H), 6.54 (t, J = 6.6 Hz, 1H, 1'-H), 5.89-6.02 (m, 1H, $CH_2CH=CH_2$), 5.30-5.39 (dm, J = 17.3 Hz, 1H, one of $CH_2CH=CH_2$), 5.20-5.27 (dm, J = 10.4 Hz, 1H, one of $CH_2CH=CH_2$), 4.48 (s, 2H, $C\equiv CCH_2$), 4.35 (m, 1H, 3'-H), 4.05-4.17 (m, 4H, $CH_2CH=CH_2$ and 5'-H), 3.99 (m, 1H, 4'-H), 2.50-2.59 (m, 2H, 2'-H); ^{31}P NMR (121.4 MHz, D_2O) δ -6.1 (d, J = 21.1 Hz, 1P, γ-P), -10.8 (d, J = 18.8 Hz, 1P, α-P), -21.9 (t, J = 19.9 Hz, 1P, β-P).

3'-*O*-Allyl-dATP-allyl-ROX (39). The procedure was similar to the synthesis of 23. The product was characterized by single base extension reaction and MALDI-TOF MS. See Fig. 10.

7. Synthesis of 3'-*O*-allyl-dGTP-allyl-Bodipy-650 (43) and 3'-*O*-allyl-dGTP-allyl-Cy5 (44)



42

3'-O-allyl-dGTP-allyl-Bodipy-650 **43**

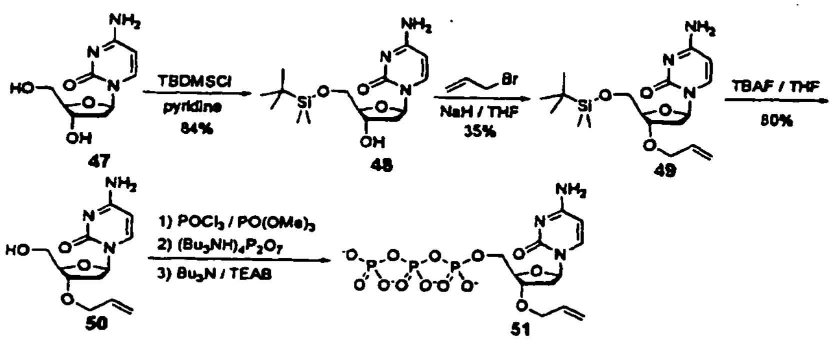
3'-O-allyl-dGTP-allyl-Cy5 **44**

Synthesis of 3'-*O*-allyl-dGTP-NH_2 42 was performed according to the procedures in reference (29).

3'-*O*-Allyl-dGTP-allyl-Bodipy-650 (43) and 3'-*O*-allyl-dGTP-allyl-Cy5 (44). The procedures were similar to the synthesis of 23. The product was characterized by single base extension reaction and MALDI-TOF MS. See Fig. 11.

II. Synthesis of 3'-*O*-allyl-dNTPs

1. Synthesis of 3'-*O*-allyl dCTP (51)



5'-*O*-(*tert*-Butyldimethylsilyl)-2'-deoxycytidine (48). To a stirred solution of 2'-deoxycytidine 47 (1.00 g; 4.40 mmol) in dry pyridine (37 mL), TBDMSCl (814 mg; 5.39 mmol) was added. The mixture was stirred at room temperature for 20 h. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:10) as the eluent to afford 48 as white solid (1.26 g; 84% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.03 (d, J = 7.5 Hz, 1H, 6-H), 6.23 (t, J = 6.3 Hz, 1H, 1'-H), 5.86 (d, J = 7.5 Hz, 1H, 5-H), 4.35 (m, 1H, 3'-H), 3.91-3.98 (m, 2H, 4'-H and one of 5'-H), 3.85 (dd, J = 2.5, 11.3 Hz, 1H, one of 5'-H), 2.36-2.43 (ddd, J = 4.1, 6.1, 13.5 Hz, 1H, one of 2'-H), 2.05-2.13 (m, 1H, one of 2'-H), 0.94 (s, 9H, $C(CH_3)_3$), 0.14 (s, 3H, one of $SiCH_3$), 0.13 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 167.1, 157.6, 142.1, 95.6, 88.7, 87.4, 71.7, 64.1, 42.7, 26.5, 19.3, -5.2, -5.3; HRMS (FAB+) calcd for $C_{15}H_{28}O_4N_3Si$ $(M+H^+)$: 342.1849, found: 342.1844.

3'-*O*-Allyl-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxycytidine (49). The procedure was similar to the synthesis of 19 and the crude product was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20)

as the eluent to afford 49 as yellow solid (480 mg; 35% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (d, J = 7.5 Hz, 1H, 6-H), 6.21 (t, J = 6.5 Hz, 1H, 1′-H), 5.87 (d, J = 7.5 Hz, 1H, 5-H), 5.87-5.97 (m, 1H, $CH_2CH{=}CH_2$), 5.26-5.33 (dm, J = 17.2 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.15-5.20 (dm, J = 10.5 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.16 (m, 1H, 3′-H), 4.11 (m, 1H, 4′-H), 3.97-4.11 (m, 2H, $CH_2CH{=}CH_2$), 3.92 (dd, J = 3.2, 11.4 Hz, 1H, one of 5′-H), 3.84 (dd, J = 2.8, 11.4 Hz, 1H, one of 5′-H), 2.46-2.51 (ddd, J = 3.1, 5.9, 13.6 Hz, 1H, one of 2′-H), 2.00-2.08 (m, 1H, one of 2′-H), 0.94 (s, 9H, $C(CH_3)_3$), 0.13 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 167.2, 157.7, 141.9, 135.6, 117.1, 95.7, 87.5, 86.6, 79.7, 71.1, 64.4, 39.8, 26.5, 19.3, -5.1, -5.2; HRMS (FAB+) calcd for $C_{15}H_{28}O_4N_3Si$ $(M{+}H^+)$: 342.1849, found: 342.1844.

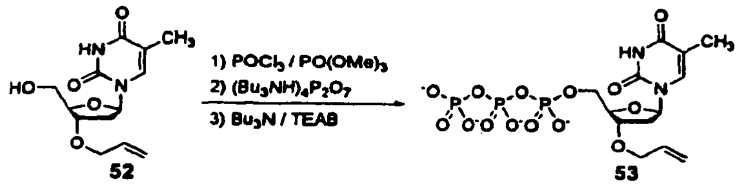
3′-*O*-Allyl-2′-deoxycytidine (50). The procedure was similar to the synthesis of 20 and the crude product was purified by flash column chromatography using CH_3OH-THF (1:12) and CH_3OH-ethyl acetate (1:4) as the eluent to afford 50 as white foam (269 mg; 80% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (d, J = 7.5 Hz, 1H, 6-H), 6.21 (dd, J = 5.9, 7.6 Hz, 1H, 1′-H), 5.89 (d, J = 7.5 Hz, 1H, 5-H), 5.87-5.98 (m, 1H, $CH_2CH{=}CH_2$), 5.27-5.33 (dm, J = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.15-5.19 (dm, J = 10.4 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.17 (m, 1H, 3′-H), 3.98-4.10 (m, 3H, 4′-H and $CH_2CH{=}CH_2$), 3.77 (dd, J = 3.6, 12.0 Hz, 1H, one of 5′-H), 3.71 (dd, J = 3.7, 12.0 Hz, 1H, one of 5′-H), 2.43-2.50 (ddd, J = 2.7, 5.9, 13.6 Hz, 1H, one of 2′-H), 2.03-2.11 (ddd, J =

6.2, 7.7, 13.6 Hz, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 167.1, 157.7, 142.2, 135.5, 117.0, 96.0, 87.5, 86.6, 80.0, 71.0, 63.0, 39.1; HRMS (FAB+) calcd for $C_{12}H_{18}O_4N_3$ ($M+H^+$): 268.1297, found: 268.1307.

3'-*O*-Allyl-2'-deoxycytidine-5'-triphosphate (51). 50 (86 mg; 0.32 mmol) and proton sponge (84 mg; 0.39 mmol) were dried in a vacuum desiccator over P_2O_5 overnight before dissolving in trimethylphosphate (0.60 mL). Freshly distilled $POCl_3$ (35.8 μL; 0.383 mmol) was added dropwise at 0 °C and the mixture was stirred for 3 h. Then the solution of tributylammonium pyrophosphate (607 mg) and tributylamine (0.61 mL; 2.56 mmol) in anhydrous DMF (2.6 mL) was well vortexed and added in one portion at room temperature and the reaction was stirred for 30 min. After that triethylammonium bicarbonate solution (TEAB) (0.1 M; 16 mL) was added and the mixture was stirred for 2 h. After most liquid was removed under vacuum, the residue was redissolved in water (2 mL) and filtered. The aqueous solution was purified by DEAE Sephadex A25 ion exchange column using gradient aqueous TEAB solution (from 0.1 M to 1.0 M) as eluent to afford 51 as colorless syrup after evaporation: ^1H NMR (300 MHz, D_2O) δ 7.90 (d, *J* = 7.4 Hz, 1H, 6-H), 6.20 (dd, *J* = 5.9, 7.6 Hz, 1H, 1'-H), 5.92 (d, *J* = 7.4 Hz, 1H, 5-H), 5.85-5.97 (m, 1H, $CH_2CH{=}CH_2$), 5.25-5.34 (m, 1H, one of $CH_2CH{=}CH_2$), 5.15-5.20 (m, 1H, one of $CH_2CH{=}CH_2$), 4.15 (m, 1H, 3'-H), 3.96-4.10 (m, 3H, 4'-H and $CH_2CH{=}CH_2$), 3.70-3.80 (m, 2H, 5'-H), 2.43-2.52 (m, 1H, one of 2'-H), 2.05-2.14 (m, 1H, one of 2'-H); ^{31}P NMR (121.4 MHz, D_2O) δ -8.8 (d, *J*

= 19.0 Hz, 1P, γ-P), -11.3 (d, *J* = 19.6 Hz, 1P, α-P), -22.9 (t, *J* = 19.5 Hz, 1P, β-P).

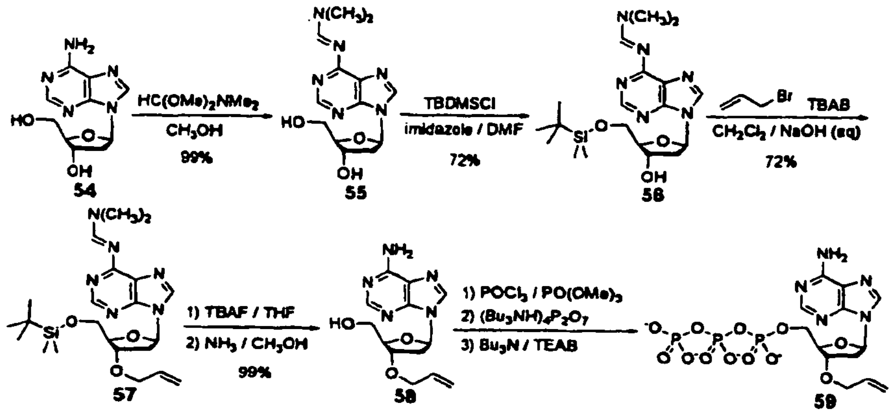
2. Synthesis of 3'-*O*-allyl-dTTP (53)



Synthesis of 3'-*O*-allylthymidine 52 was performed according to the procedures in reference (28).

3'-*O*-Allylthymidine-5'-triphosphate (53). The procedure was similar to the synthesis of 51 to yield 53 as colorless syrup: ^{1}H NMR (300 MHz, D_2O) δ 7.80 (m, 1H, 6-H), 6.23 (dd, *J* = 6.2, 8.1 Hz, 1H, 1'-H), 5.85-5.97 (m, 1H, $CH_2CH{=}CH_2$), 5.25-5.32 (m, 1H, one of $CH_2CH{=}CH_2$), 5.15-5.21 (m, 1H, one of $CH_2CH{=}CH_2$), 4.17 (m, 1H, 3'-H), 3.97-4.11 (m, 3H, 4'-H and $CH_2CH{=}CH_2$), 3.70-3.80 (m, 2H, 5'-H), 2.30-2.41 (m, 1H, one of 2'-H), 2.11-2.23 (m, 1H, one of 2'-H), 1.86 (d, *J* = 1.2 Hz, 3H, CH_3); ^{31}P NMR (121.4 MHz, D_2O) δ -7.1 (d, *J* = 20.1 Hz, 1P, γ-P), -10.8 (d, *J* = 19.5 Hz, 1P, α-P), -21.8 (t, *J* = 19.5 Hz, 1P, β-P).

3. Synthesis of 3'-*O*-allyl-dATP (59)



N^6-[(Dimethylamino)methylene]-2′-deoxyadenosine (55). To a stirred solution of 2′-deoxyadenosine monohydrate 54 (1.00 g; 3.71 mmol) in methanol (43 mL), *N,N*-dimethylformamide dimethyl acetal (2.48 mL; 18.6 mmol) was added. The reaction was stirred at 50 °C for 16 h. After evaporation, CH_2Cl_2-hexane (1:1) was added. The white solid formed was then filtered, collected and washed by hexane to afford 55 as white solid (1.13 g; 99% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.92 (s, 1H, $C\mathit{H}N(CH_3)_2$), 8.44 (s, 1H, 2-H), 8.43 (s, 1H, 8-H), 6.48 (dd, *J* = 6.2, 7.8 Hz, 1H, 1′-H), 4.59 (m, 1H, 3′-H), 4.07 (m, 1H, 4′-H), 3.86 (dd, *J* = 3.1, 12.2 Hz, 1H, one of 5′-H), 3.76 (dd, *J* = 3.5, 12.2 Hz, 1H, one of 5′-H), 3.25 (s, 3H, one of $NC\mathit{H}_3$), 3.24 (s, 3H, one of $NC\mathit{H}_3$), 2.80-2.88 (ddd, *J* = 5.9, 7.8, 13.5 Hz, 1H, one of 2′-H), 2.40-2.47 (ddd, *J* = 2.8, 6.1, 13.4 Hz, 1H, one of 2′-H); ^{13}C NMR (100 MHz, CD_3OD) δ 161.0, 159.9, 152.8, 151.1, 142.8, 127.0, 89.7, 86.9, 72.9, 63.5, 41.5 ($N(\mathit{C}H_3)_2$), 35.3; HRMS (FAB+) calcd for $C_{13}H_{19}O_3N_6$ $(M+H^+)$: 307.1519, found: 307.1511.

5′-*O*-(*tert*-Butyldimethylsilyl)-N^6-[(dimethylamino)methylene]-2′-deoxyadenosine (56). The procedure was similar to the synthesis of 18, and the crude product was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20) as the eluent to afford 56 as white foam (1.11 g; 72% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.90 (s, 1H, C*H*N(CH_3)$_2$), 8.45 (s, 1H, 2-H), 8.43 (s, 1H, 8-H), 6.49 (t, *J* = 6.5 Hz, 1H, 1′-H), 4.59 (m, 1H, 3′-H), 4.04 (m, 1H, 4′-H), 3.95 (dd, *J* = 3.7, 11.3 Hz, 1H, one of 5′-H), 3.85 (dd, *J* = 2.8, 11.3 Hz, 1H, one of 5′-H), 3.25 (s, 3H, one of NC*H*$_3$), 3.24 (s, 3H, one of NC*H*$_3$), 2.73-2.81 (m, 1H, one of 2′-H), 2.48-2.55 (ddd, *J* = 4.0, 6.4, 13.5 Hz, 1H, one of 2′-H), 0.90 (s, 9H, C(C*H*$_3$)$_3$), 0.08 (s, 3H, one of SiC*H*$_3$), 0.07 (s, 3H, one of SiC*H*$_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 160.6, 159.7, 153.0, 151.7, 141.8, 126.5, 88.9, 85.7, 72.1, 64.3, 41.6, 41.5, 35.3, 26.5, 19.3, -5.0, -5.1; HRMS (FAB+) calcd for $C_{19}H_{33}O_3N_6Si$ (M+H$^+$): 421.2383, found: 421.2390.

3′-*O*-Allyl-5′-*O*-(t-butyldimethylsilyl)-N^6-[(dimethylamino)methylene]-2′-deoxyadenosine (57). The procedure was similar to the synthesis of 35, and the crude product was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:25) and CH_3OH-ethyl acetate (1:10) as the eluent to afford 57 as colorless oil (875 mg; 72% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.90 (s, 1H, C*H*N(CH_3)$_2$), 8.44 (s, 1H, 2-H), 8.41 (s, 1H, 8-H), 6.45 (dd, *J* = 6.3, 7.2 Hz, 1H, 1′-H), 5.91-6.01 (m, 1H, CH_2C*H*=CH_2), 5.30-5.37 (dm, *J* = 17.2 Hz, 1H, one of CH_2CH=C*H*$_2$), 5.18-5.22 (dm, *J* = 10.5 Hz, 1H, one of

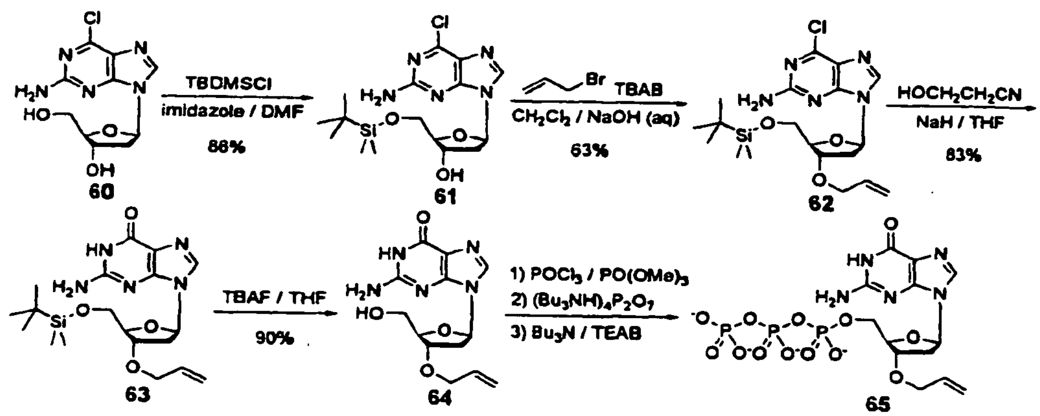
$CH_2CH=CH_2$), 4.37 (m, 1H, 3'-H), 4.17 (m, 1H, 4'-H), 4.05-4.15 (m, 2H, $CH_2CH=CH_2$), 3.91 (dd, J = 4.6, 11.1 Hz, 1H, one of 5'-H), 3.83 (dd, J = 3.8, 11.1 Hz, 1H, one of 5'-H), 3.25 (s, 3H, one of NCH_3), 3.24 (s, 3H, one of NCH_3), 2.76-2.83 (ddd, J = 6.0, 7.3, 13.6 Hz, 1H, one of 2'-H), 2.59-2.65 (ddd, J = 3.0, 6.1, 13.6 Hz, 1H, one of 2'-H), 0.90 (s, 9H, $C(CH_3)_3$), 0.08 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 160.7, 159.7, 153.1, 151.8, 141.9, 135.6, 126.5, 117.1, 86.7, 85.9, 80.1, 71.1, 64.5, 41.5, 38.7, 35.3, 26.5, 19.3, -5.0, -5.1; HRMS (FAB+) calcd for $C_{22}H_{37}O_3N_6Si$ ($M+H^+$): 461.2696, found: 461.2695.

3'-O-Allyl-2'-deoxyadenosine (58). To a stirred solution of 57 (875 mg; 1.90 mmol) in anhydrous THF (45 mL), 1 M TBAF in THF solution (2.09 mL; 2.09 mmol) was added and the reaction was stirred at room temperature for 1 h. After evaporation, the residue was dissolved in 7 M ammonia in methanol solution (34 mL). The mixture was then stirred in a sealed flask at 50 °C for 9 h. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:10) as the eluent to afford 58 as white solid (548 mg; 99% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.30 (s, 1H, 2-H), 8.17 (s, 1H, 8-H), 6.38 (dd, J = 5.8, 8.6 Hz, 1H, 1'-H), 5.91-6.01 (m, 1H, $CH_2CH=CH_2$), 5.30-5.37 (dm, J = 17.3 Hz, 1H, one of $CH_2CH=CH_2$), 5.17-5.22 (dm, J = 10.6 Hz, 1H, one of $CH_2CH=CH_2$), 4.36 (m, 1H, 3'-H), 4.21 (m, 1H, 4'-H), 4.04-4.15 (m, 2H, $CH_2CH=CH_2$), 3.85 (dd, J = 3.2, 12.3 Hz, 1H, one of 5'-H), 3.74 (dd, J = 3.2, 12.3 Hz, 1H, one of 5'-H), 2.75-2.83 (ddd, J =

5.7, 8.6, 13.6 Hz, 1H, one of 2'-H), 2.52-2.58 (ddd, J = 1.8, 5.8, 13.6 Hz, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.1, 153.1, 149.5, 141.2, 135.6, 120.6, 117.0, 87.5, 87.2, 80.9, 71.0, 63.9, 38.7; HRMS (FAB+) calcd for $C_{13}H_{18}O_3N_5$ $(M+H^+)$: 292.1410, found: 292.1426.

3'-O-Allyl-2'-deoxyadenosine-5'-triphosphate (59). The procedure was similar to the synthesis of 51 to yield 59 as colorless syrup: ^{1}H NMR (300 MHz, D_2O) δ 8.46 (s, 1H, 2-H), 8.19 (s, 1H, 8-H), 6.43 (dd, J = 6.3, 7.2 Hz, 1H, 1'-H), 5.90-6.02 (m, 1H, $CH_2CH{=}CH_2$), 5.31-5.40 (dm, J = 17.1 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.21-5.28 (dm, J = 10.8 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.55 (m, 1H, 3'-H), 4.40 (m, 1H, 4'-H), 4.06-4.20 (m, 4H, $CH_2CH{=}CH_2$ and 5'-H), 2.61-2.82 (m, 2H, 2'-H) ; ^{31}P NMR (121.4 MHz, D_2O) δ -8.9 (d, J = 19.1 Hz, 1P, γ-P), -11.2 (d, J = 19.7 Hz, 1P, α-P), -22.8 (t, J = 19.9 Hz, 1P, β-P).

4. Synthesis of 3'-O-allyl-dGTP (65)



2-Amino-6-chloro-9-[*β*-D-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxyribofuranosyl] purine (61). The procedure was similar to the synthesis of 18, and the crude product was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20) as the eluent to afford 61 as white solid (1.20 g; 86% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.25 (s, 1H, 8-H), 6.34 (t, *J* = 6.4 Hz, 1H, 1'-H), 4.56 (m, 1H, 3'-H), 4.01 (m, 1H, 4'-H), 3.90 (dd, *J* = 3.5, 11.4 Hz, 1H, one of 5'-H), 3.84 (dd, *J* = 3.8, 11.4 Hz, 1H, one of 5'-H), 2.67-2.74 (m, 1H, one of 2'-H), 2.43-2.50 (ddd, *J* = 4.2, 6.4, 13.5 Hz, 1H, one of 2'-H), 0.89 (s, 9H, $C(CH_3)_3$), 0.07 (s, 3H, one of $SiCH_3$), 0.06 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 161.1, 154.2, 151.2, 142.0, 124.9, 88.9, 85.5, 72.0, 64.3, 41.4, 26.5, 19.3, -5.1 (two $SiCH_3$); HRMS (FAB+) calcd for $C_{16}H_{27}O_3N_5ClSi$ $(M+H^+)$: 400.1572, found: 400.1561.

2-Amino-6-chloro-9-[*β*-D-3'-*O*-allyl-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxyribo-furanosyl]-purine (62). The procedure was the same as that of 35, and the crude product 61 converted from 60 was purified by flash column chromatography using ethyl acetate-hexane (1:2) as the eluent to afford 62 as white solid (832 mg; 63% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.23 (s, 1H, 8-H), 6.30 (t, *J* = 6.7 Hz, 1H, 1'-H), 5.89-5.99 (m, 1H, $CH_2CH{=}CH_2$), 5.28-5.35 (dm, *J* = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.16-5.21 (dm, *J* = 10.5 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.33 (m, 1H, 3'-H), 4.13 (m, 1H, 4'-H), 4.03-4.12 (m, 2H, $CH_2CH{=}CH_2$), 3.86 (dd, *J* = 4.3, 11.2 Hz, 1H, one of 5'-H), 3.81 (dd, *J* = 3.9, 11.2 Hz, 1H,

one of 5'-H), 2.68-2.75 (m, 1H, one of 2'-H), 2.53-2.59 (ddd, J = 3.2, 6.2, 13.6 Hz, 1H, one of 2'-H), 0.88 (s, 9H, $C(CH_3)_3$), 0.08 (s, 3H, one of $SiCH_3$), 0.07 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 161.1, 154.2, 151.2, 141.9, 135.5, 124.9, 117.1, 86.7, 85.6, 80.0, 71.1, 64.5, 38.7, 26.5, 19.3, -5.1, -5.2; HRMS (FAB+) calcd for $C_{19}H_{31}O_3N_5ClSi$ $(M+H^+)$: 440.1885, found: 440.1870.

3'-*O*-Allyl-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxyguanosine (63). To a stirred suspension of 95% NaH power (223 mg; 8.83 mmol) in anhydrous THF (82 mL), 3-hydroxypropionitrile (550 μL; 8.00 mmol) was added and the mixture was stirred at room temperature for 20 min. Then 62 (832 mg; 1.89 mmol) in anhydrous THF (20 mL) was added and the mixture was stirred at 40 °C for 1 h. At room temperature, 80% acetic acid (630 μL; 8.83mmol) was added and stirred for 20 min. After evaporation, ethyl acetate (100 mL) was added. The mixture was washed by saturated aqueous $NaHCO_3$, NaCl, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:20) as the eluent to afford 63 as white solid (661 mg; 83% yield): ^1H NMR (400 MHz, CD_3OD) δ 7.92 (s, 1H, 8-H), 6.22 (dd, J = 6.4, 7.3 Hz, 1H, 1'-H), 5.89-5.99 (m, 1H, $CH_2CH{=}CH_2$), 5.29-5.35 (dm, J = 17.3 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.17-5.21 (dm, J = 10.5 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.30 (m, 1H, 3'-H), 4.11 (m, 1H, 4'-H), 4.03-4.12 (m, 2H, $CH_2CH{=}CH_2$), 3.79-3.86 (m, 2H, 5'-H), 2.56-2.64 (ddd, J = 5.9, 7.4, 13.5 Hz, 1H, one of 2'-H), 2.49-2.55 (ddd, J = 3.0, 6.1, 13.5 Hz,

1H, one of 2'-H), 0.91 (s, 9H, $C(CH_3)_3$), 0.10 (s, 3H, one of $SiCH_3$), 0.09 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.7, 153.4, 151.0, 135.3, 134.1, 117.2, 117.1, 85.0, 83.8, 78.7, 70.2, 63.3, 38.2, 26.1, 18.5, -5.1, -5.3; HRMS (FAB+) calcd for $C_{19}H_{32}O_4N_5Si$ $(M+H^+)$: 422.2224, found: 422.2209.

3'-*O*-Allyl-2'-deoxyguanosine (64). The procedure was similar to the synthesis of 20 and the crude product was purified by flash column chromatography using CH_3OH-CH_2Cl_2 (1:10) as the eluent to afford 64 as white solid (434 mg; 90% yield): ^1H NMR (400 MHz, CD_3OD) δ 7.94 (s, 1H, 8-H), 6.22 (dd, *J* = 5.9, 8.4 Hz, 1H, 1'-H), 5.90-6.00 (m, 1H, $CH_2CH{=}CH_2$), 5.29-5.36 (dm, *J* = 17.2 Hz, 1H, one of $CH_2CH{=}CH_2$), 5.17-5.21 (dm, *J* = 10.5 Hz, 1H, one of $CH_2CH{=}CH_2$), 4.31 (m, 1H, 3'-H), 4.14 (m, 1H, 4'-H), 4.03-4.13 (m, 2H, $CH_2CH{=}CH_2$), 3.80 (dd, *J* = 3.8, 12.0 Hz, 1H, one of 5'-H), 3.72 (dd, *J* = 3.7, 12.0 Hz, 1H, one of 5'-H), 2.63-2.71 (ddd, *J* = 5.9, 8.4, 13.6 Hz, 1H, one of 2'-H), 2.45-2.52 (ddd, *J* = 2.1, 5.9, 13.6 Hz, 1H, one of 2'-H); ^{13}C NMR (100 MHz, DMSO-d6) δ 156.4, 153.4, 150.6, 135.1, 134.8, 116.5, 116.3, 84.9, 82.6, 79.1, 69.0, 61.8, 36.4; HRMS (FAB+) calcd for $C_{13}H_{18}O_4N_5$ $(M+H^+)$: 308.1359, found: 308.1358.

3'-*O*-Allyl-2'-deoxyguanosine-5'-triphosphate (65). The procedure was similar to the synthesis of 51 to yield 65 as colorless syrup: ^1H NMR (300 MHz, D_2O) δ 7.90 (s, 1H, 8-H), 6.21 (dd, *J* = 6.1, 8.1 Hz, 1H, 1'-H), 5.86-5.96 (m, 1H, $CH_2CH{=}CH_2$), 5.27-5.35 (m, 1H, one of $CH_2CH{=}CH_2$), 5.15-5.20 (m, 1H, one of $CH_2CH{=}CH_2$), 4.30 (m,

1H, 3'-H), 4.15 (m, 1H, 4'-H), 4.02-4.14 (m, 2H, $CH_2CH=CH_2$), 3.75-3.85 (m, 2H, 5'-H), 2.60-2.73 (m, 1H, one of 2'-H), 2.42-2.50 (m, 1H, one of 2'-H); ^{31}P NMR (121.4 MHz, D_2O) δ -10.9 (d, J = 18.9 Hz, 1P, γ-P), -11.3 (d, J = 19.6 Hz, 1P, α-P), -22.9 (t, J = 19.6 Hz, 1P, β-P).

III. Construction of a Chip with Immobilized Self-priming DNA Template.

The DNA chip was constructed as shown in Fig.5 and involved the following three steps:

Synthesis of the alkyne-functionalized DNA template. The 5'-amino-hairpin DNA templates (GeneLink, NY) 5'-NH_2-TTT-TTG-TTT-TTT-TTT-TCG-ATC-GAC-TTA-AGG-CGC-TTG-CGC-CTT-AAG-TCG-3' and 5'-NH_2-AGT-CAG-TCT-CTC-ATC-TCG-ACA-TCT-ACG-CTA- CTC-GTC-GAT-CGG-AAA-CAG-CTA-TGA-CCA-TGC-TTG-CAT-GGT-CAT-AGC-TGT-TTC-C-3' were coupled with 6-heptynoic acid by adding 300 μL DMSO solution of 6-heptynoic-NHS ester [succinimidyl N-(6-heptynoate)] (0.8 M) into the 1000 μL DNA template solution (200 μM, in 0.25 M $Na_2CO_3/NaHCO_3$ buffer, pH 9.0). The reaction mixture was stirred for 5 h at room temperature to produce a terminal alkynyl group on the 5'-end of the hairpin DNA. The resulting alkyne-functionalized DNA was separated from the excess reagent by size-exclusion chromatography using PD-10 columns (GE Health, NJ). Further desalting with an oligonucleotide purification cartridge (Applied Biosystems, CA) and drying afforded the crude product which was further purified by reverse-phase HPLC (Waters HPLC system

containing Waters Delta 600 controller, Rheodyne 7725i injector and 2996 photodiode array detector, Milford, MA) using a C-18 reverse column (Xterra MS C18, 4.6 mm x 50 mm, 2.5 μm) at a flow rate of 0.5 mL/min, with detection at 260 nm, and elution with a linear gradient of 12-34.5% of B in A over 40 min (A: 4.4 mM triethylamine and 100 mM hexafluoroisopropyl alcohol aqueous solution, pH 8.1; B: methanol). The fractions containing the desired product were collected and evaporated to dryness under vacuum. MALDI-TOF MS was used to characterize the product on a Voyager DE matrix assisted laser spectrometer (Applied Biosystems, CA) using 3-hydroxypicolinic acid as a matrix.

Azide functionalization of an amine-modified glass surface. The amine-modified glass slide (Corning® GAPS II) was cleaned and pre-treated by immersion into a basic solution [N,N-diisopropyl ethylamine (DIPEA)/dimethylformamide (DMF), 1:9 (V/V)] for 30 min. The glass slide was then washed with DMF, and transferred into the 2 mL DMF coupling solution containing 100 mM O-(2-azidoethyl)-O′-[2-(diglycolyl-amino)-ethyl]heptaethylene glycol (Fluka, Switzerland), Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (Novabiochem, CA) and 200 mM DIPEA. The reaction vessel was gently shaken for 4 h at room temperature. The azide functionalized glass slide was washed thoroughly with DMF and ethanol, and then dried under argon gas stream.

DNA immobilization on the azide-modified glass surface using 1,3-dipolar alkyne-azide cycloaddition chemistry.

A coupling mixture was prepared by mixing *tetrakis*-(acetonitrile)copper (I) hexafluorophosphate (2 mM/DMSO), *tris*-(benzyltriazolylmethyl) amine (TBTA) (2 mM/DMSO), sodium ascorbate (2.6 mM/H_2O) and alkynyl DNA (50 µM/H_2O) with a volumetric ratio of 3:3:2.3:3. This coupling mixture was then spotted onto the azide-functionalized glass slide in the form of 11.0 µL drops with the aid of adhesive silicone isolators (Grace Bio-Labs, OR) to create uniform spots on the glass surface. The DNA spotted glass slide was incubated in a humid chamber at room temperature for 8 h, then washed with de-ionized water and SPSC buffer (50 mM sodium phosphate/1 M NaCl, pH 7.5) for 1/2 h to remove non-specifically bound DNA, and finally rinsed with dH_2O. The formation of a stable hairpin was ascertained by covering the DNA spots with 1X Thermolpol II reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 0.1% Triton X-100, 4 mM $MnCl_2$, pH 8.8), incubating it in a humid chamber at 95 °C for 5 min to dissociate any partial hairpin structure, and then cooling slowly for re-annealing.

IV. Continuous DNA polymerase reaction using four chemically cleavable fluorescent nucleotides as reversible terminators in solution.

We characterized the four nucleotide analogues 3'-O-*allyl*-dCTP-*allyl*-Bodipy-FL-510, 3'-O-*allyl*-dUTP-*allyl*-R6G, 3'-O-*allyl*-dATP-*allyl*-ROX and 3'-O-*allyl*-dGTP-*allyl*-Bodipy-650, by performing four continuous DNA-extension reactions sequentially using a primer (5'-AGAGGATCCAACCGAGAC-3') and a synthetic DNA template

(5'-GTGTACATCAACATCACCTACCACCATGTCAGTCTCGGTTGGATCCTCTATTGTGTCCGG-3') based on a portion of exon 7 of the human *p53* gene. The four nucleotides in the template immediately adjacent to the annealing site of the primer are 3'-ACTG-5'. First, a polymerase extension reaction using a pool of all four nucleotide analogues along with the primer and the template was performed producing a single base extension product. The reaction mixture for this, and all subsequent extension reactions, consisted of 80 pmol of template, 50 pmol of primer, 100 pmol of 3'-O-*allyl*-dNTPs-*allyl*-fluorophore, 1X Thermopol II reaction buffer, 40 nmol of Mn^{2+} and 2 U of 9°N mutant DNA polymerase (exo-) A485L/Y409V in a total volume of 20 µL. The reaction consisted of 20 cycles at 94°C for 20 sec, 48°C for 40 sec, and 62°C for 90 sec. Subsequently, the extension product was purified by using reverse-phase HPLC. The fraction containing the desired DNA product was collected and freeze-dried for analysis using MALDI-TOF mass spectrometry. For deallylation, the purified DNA extension product bearing the fluorescent nucleotide analogue was resuspended in degassed water and added to a deallylation cocktail [1X Thermopol I reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$] and incubated for 30 s to yield deallylated DNA product which was characterized by MALDI-TOF MS. The DNA product with both the fluorophore and the 3'-O-allyl group removed to generate a free 3'-OH group was used as a primer for a second extension reaction using 3'-O-*allyl*-dNTPs-*allyl*-fluorophore. The second extended DNA

product was then purified by HPLC and deallylated. The third and the fourth extensions were carried out in a similar manner using the previously extended and deallylated product as the primer.

V. 4-Color SBS reaction on a chip with four chemically cleavable fluorescent nucleotides as reversible terminators.

Ten microliters of a solution consisting of 3'-O-*allyl*-dCTP-*allyl*-Bodipy-FL-510 (3 pmol), 3'-O-*allyl*-dUTP-*allyl*-R6G (10 pmol), 3'-O-*allyl*-dATP-*allyl*-ROX (5 pmol) and 3'-O-*allyl*-dGTP-*allyl*-Cy5 (2 pmol), 1 U of 9°N mutant DNA polymerase, and 1X Thermolpol II reaction buffer was spotted on the surface of the chip, where the self-primed DNA moiety was immobilized. The nucleotide analogue complementary to the DNA template was allowed to incorporate into the primer at 68°C for 10 min. To synchronize any unincorporated templates, an extension solution consisting of 30 pmol each of 3'-O-allyl-dCTP, 3'-O-allyl-dTTP, 3'-O-allyl-dATP and 3'-O-allyl-dGTP, 1 U of 9°N mutant DNA polymerase, and 1X Thermolpol II reaction buffer was spotted on the same spot and incubated at 68°C for 10 min. After washing the chip with a SPSC buffer containing 0.1% Tween 20 for 5 min, the surface was rinsed with dH_2O, dried briefly and then scanned with a 4-color ScanArray Express scanner (Perkin-Elmer Life Sciences) to detect the fluorescence signal. The 4-color scanner is equipped with four lasers with excitation wavelengths of 488, 543, 594, and 633 nm and emission filters centered at 522, 570, 614, and 670 nm. For deallylation, the chip was immersed in a deallylation

cocktail [1X Thermolpol I reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$] and incubated for 5 min at 60 °C. The chip was then immediately immersed in a 3 M Tris-HCl buffer (pH 8.5) and incubated for 5 min at 60 °C. Finally, the chip was rinsed with acetonitrile/dH_2O (1:1, V/V) and dH_2O. The chip surface was scanned again to compare the intensity of fluorescence after deallylation with the original fluorescence intensity. This process was followed by the next polymerase extension reaction using 3'-O-*allyl*-dNTPs-*allyl*-fluorophore and 3'-O-allyl-dNTPs, with the subsequent washing, fluorescence detection, and deallylation processes performed as described above. The same cycle was repeated multiple times using the four chemically cleavable fluorescent nucleotide mixture in polymerase extension reaction to obtain *de novo* DNA sequencing data on various different DNA templates.

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What is claimed is:

1. A method for determining the sequence of a DNA comprising performing the following steps for each residue of the DNA to be sequenced:
   (a) contacting the DNA with a DNA polymerase in the presence of (i) a primer and (ii) four nucleotide analogues under conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (1) the nucleotide analogues consist of an analogue of dGTP, an analogue of dCTP, an analogue of dTTP or dUTP, and an analogue of dATP, (2) each nucleotide analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, and analogues thereof, (ii) a deoxyribose, (iii) a moiety cleavably linked to the 3'-oxygen of the deoxyribose and (iv) a unique label cleavably linked to the base, so that a nucleotide analogue complementary to the residue being sequenced is incorporated into the DNA by the DNA polymerase, and (3) each of the four analogues has a unique label which is different than the unique labels of the other three analogues;
   (b) removing unbound nucleotide analogues;
   (c) again contacting the DNA with a DNA polymerase in the presence of (i) a primer and (ii) four reversible terminators under conditions permitting the DNA polymerase to catalyze DNA synthesis, wherein (1) the reversible terminators consist of an analogue of dGTP, an analogue of dCTP, an analogue of dTTP or dUTP, and an

analogue of dATP, (2) each nucleotide analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, and analogues thereof, which base does not have a unique label bound thereto, (ii) a deoxyribose, and (iii) a moiety cleavably linked to the 3'-oxygen of the deoxyribose;

(d) removing unbound reversible terminators;

(e) determining the identity of the nucleotide analogue incorporated in step (a) via determining the identity of the corresponding unique label, with the proviso that step (e) can either precede step (c) or follow step (d); and

(f) following step (e), except with respect to the final DNA residue to be sequenced, cleaving from the incorporated nucleotide analogues the unique label, if applicable, and the moiety linked to the 3'-oxygen atom of the deoxyribose,

thereby determining the sequence of the DNA.

2. The method of claim 1, wherein step (e) is performed before step (c).

3. The method of claim 1, wherein the moiety cleavably linked to the 3'-oxygen of the deoxyribose is chemically cleavable or photocleavable.

4. The method of claim 1, wherein the moiety cleavably linked to the 3'-oxygen of the deoxyribose in the nucleotide analogs of step (a) is an allyl moiety or a 2-nitrobenzyl moiety.

5. The method of claim 1, wherein the moiety cleavably linked to the 3'-oxygen of the deoxyribose in the reversible terminators of step (c) is an allyl moiety or a 2-nitrobenzyl moiety.

6. The method of claim 1, wherein the unique label is bound to the base via a chemically cleavable or photocleavable linker.

7. The method of claim 1, wherein the unique label bound to the base via a cleavable linker is a dye, a fluorophore, a chromophore, a combinatorial fluorescence energy transfer tag, a mass tag, or an electrophore.

8. The method of claim 3, wherein the moiety is chemically cleavable with $Na_2PdCl_4/P(PhSO_3Na)_3$.

9. The method of claim 6, wherein the linker is chemically cleavable with $Na_2PdCl_4/P(PhSO_3Na)_3$.

10. The method of claim 1, wherein the primer is a self-priming moiety.

11. The method of claim 1, wherein the DNA is bound to a solid substrate.

12. The method of claim 11, wherein the DNA is bound to the solid substrate via 1,3-dipolar azide-alkyne cycloaddition chemistry.

13. The method of claim 11, wherein the DNA is bound to the solid substrate via a polyethylene glycol molecule.

14. The method of claim 13, wherein the DNA is alkyne-labeled.

15. The method of claim 11, wherein the DNA is bound to the solid substrate via a polyethylene glycol molecule and the solid substrate is azide-functionalized.

16. The method of claim 11, wherein the DNA is immobilized on the solid substrate via an azido linkage, an alkynyl linkage, or biotin-streptavidin interaction.

17. The method of claim 11, wherein the solid substrate is in the form of a chip, a bead, a well, a capillary tube, a slide, a wafer, a filter, a fiber, a porous media, or a column.

18. The method of claim 11, wherein the solid substrate is gold, quartz, silica, plastic, glass, diamond, silver, metal, or polypropylene.

19. The method of claim 11, wherein the solid substrate is porous.

20. The method of claim 11, wherein about 1000 or fewer copies of the DNA are bound to the solid substrate.

21. The method of claim 1, wherein the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Cy5,

3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

22. The method of claim 1, wherein the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-FL-510, 3'-O-allyl-dCTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

23. The method of claim 1, wherein the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

24. The method of claim 1, wherein the reversible terminators in step (c) are 3'-O-allyl-dGTP, 3'-O-allyl-dCTP, 3'-O-allyl-dATP and 3'-O-allyl-dUTP.

25. The method of claim 1, wherein the reversible terminators in step (c) are 3'-O-2-nitrobenzyl-dGTP, 3'-O-2-nitrobenzyl-dCTP, 3'-O-2-nitrobenzyl-dATP and 3'-O-2-nitrobenzyl-dUTP.

26. The method of claim 1, wherein the DNA polymerase is a 9°N polymerase or a variant thereof.

27. The method of claim 1, wherein the DNA is bound to the solid substrate via a polyethylene glycol molecule and the solid substrate is azide-functionalized or the DNA is immobilized on the solid substrate via an azido linkage, an alkynyl linkage, or biotin-streptavidin interaction;

wherein (i) the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Cy5, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP- allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G, (ii) the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-FL-510, 3'-O-allyl-dCTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G, or (iii) the four nucleotide analogues in step (a) are 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G; and
wherein the reversible terminators in step (c) are 3'-O-allyl-dGTP, 3'-O-allyl-dCTP, 3'-O-allyl-dATP and 3'-O-allyl-dUTP.

28. A kit for performing the method of claim 1, comprising, in separate compartments,
   (a) nucleotide analogues of (i) GTP, (ii) ATP, (iii) CTP and (iv) TTP or UTP, wherein each analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, or an analogue thereof, (ii) a deoxyribose, (iii) a cleavable moiety bound to the 3'-oxygen of the deoxyribose and (iv) a unique label bound to the base via a cleavable linker,
   (b) reversible terminators comprising a nucleotide analogue of (i) GTP, (ii) ATP, (iii) CTP and (iv) TTP or UTP, wherein each analogue comprises (i) a base selected from the group consisting of adenine, guanine, cytosine, thymine or uracil, or an analogue thereof, which base does not have a

unique label bound thereto, (ii) a deoxyribose, and (iii) a cleavable moiety bound to the 3'-oxygen of the deoxyribose;

(c) reagents suitable for use in DNA polymerization; and

(d) instructions for use.

29. The kit of claim 28, wherein the nucleotide analogues of part (a) are 3'-O-allyl-dGTP-allyl-Cy5, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP- allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

30. The kit of claim 28, wherein the nucleotide analogues of part (a) are 3'-O-allyl-dGTP-allyl-Bodipy-FL-510, 3'-O-allyl-dCTP-allyl-Bodipy-650, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

31. The kit of claim 28, wherein the nucleotide analogues of part (a) are 3'-O-allyl-dGTP-allyl-Bodipy-650, 3'-O-allyl-dCTP-allyl-Bodipy-FL-510, 3'-O-allyl-dATP-allyl-ROX and 3'-O-allyl-dUTP-allyl-R6G.

32. The kit of claim 28, wherein the nucleotide analogues of part (b) are 3'-O-allyl-dGTP, 3'-O-allyl-dCTP, 3'-O-allyl-dATP and 3'-O-allyl-dUTP.

33. The method of claim 28, wherein the reversible terminators in step (c) are 3'-O-2-nitrobenzyl-dGTP, 3'-O-2-nitrobenzyl-dCTP, 3'-O-2-nitrobenzyl-dATP and 3'-O-2-nitrobenzyl-dUTP.

Figure 1

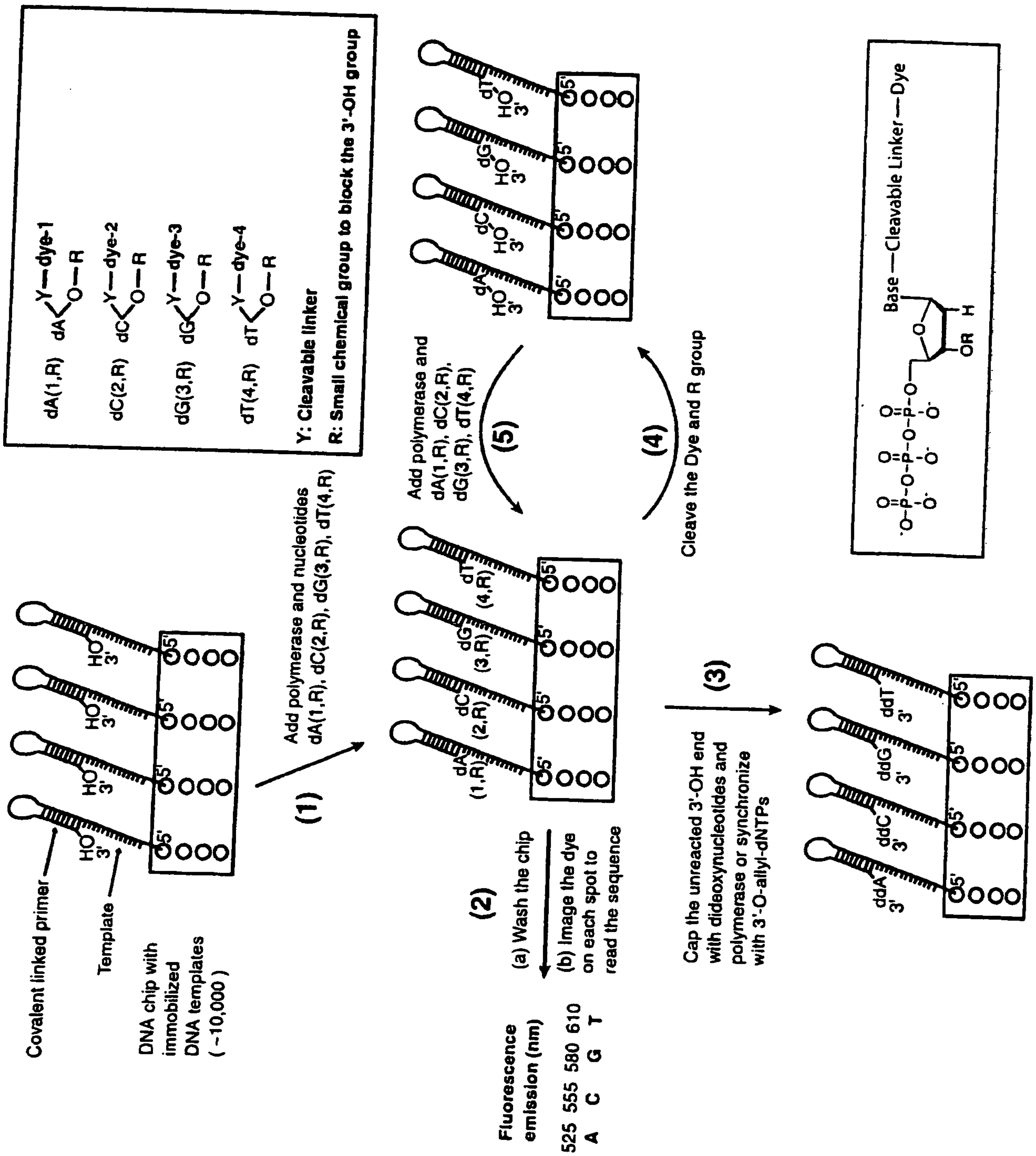
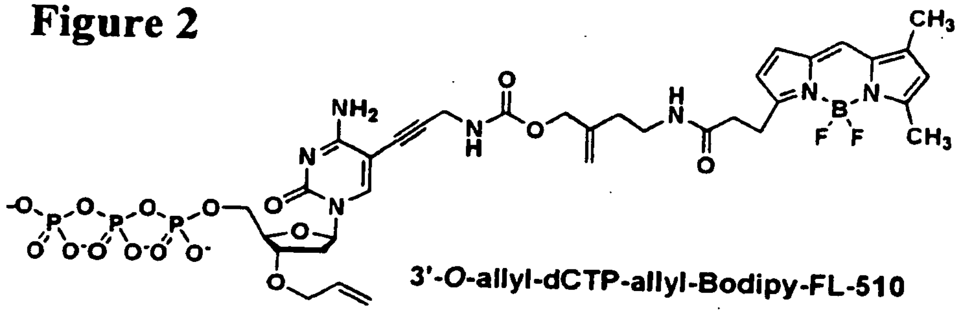
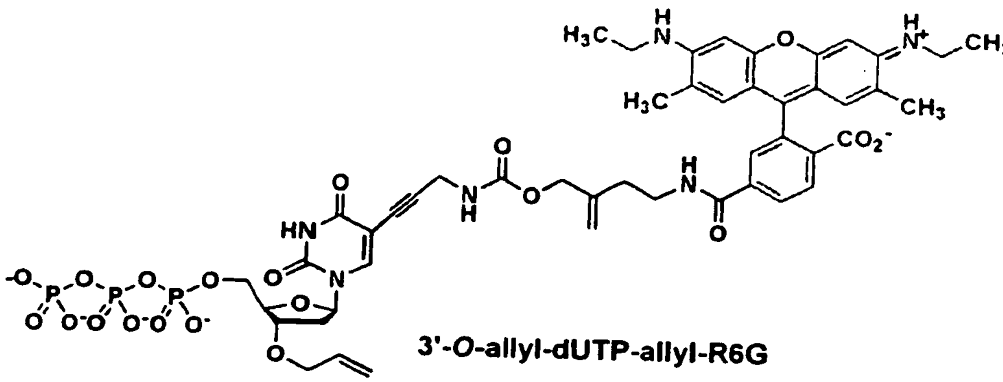

dA(1,R) dA<Y—dye-1 O—R
dC(2,R) dC<Y—dye-2 O—R
dG(3,R) dG<Y—dye-3 O—R
dT(4,R) dT<Y—dye-4 O—R
Y: Cleavable linker
R: Small chemical group to block the 3'-OH group
Covalent linked primer
Template
DNA chip with immobilized DNA templates (~10,000)
(1)
Add polymerase and nucleotides dA(1,R), dC(2,R), dG(3,R), dT(4,R)
(2)
(a) Wash the chip
(b) Image the dye on each spot to read the sequence
Fluorescence emission (nm)
525 555 580 610
A C G T
(3)
Cap the unreacted 3'-OH end with dideoxynucleotides and polymerase or synchronize with 3'-O-allyl-dNTPs
(4)
Cleave the Dye and R group
(5)
Add polymerase and dA(1,R), dC(2,R), dG(3,R), dT(4,R)
Base —Cleavable Linker—Dye
OR
H

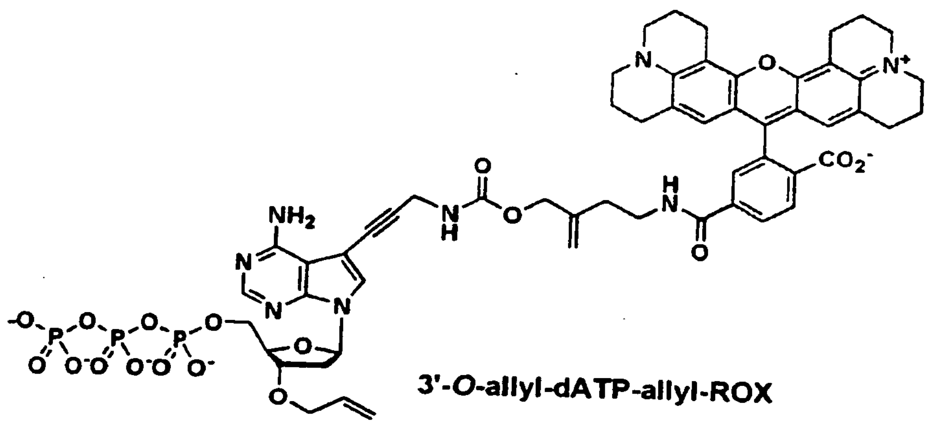
Figure 2



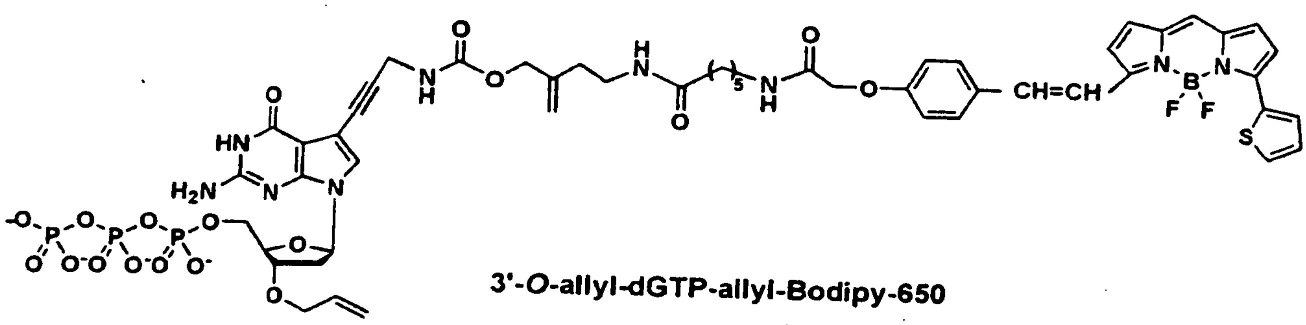
3'-*O*-allyl-dCTP-allyl-Bodipy-FL-510



3'-*O*-allyl-dUTP-allyl-R6G



3'-*O*-allyl-dATP-allyl-ROX



3'-*O*-allyl-dGTP-allyl-Bodipy-650

Figure 3

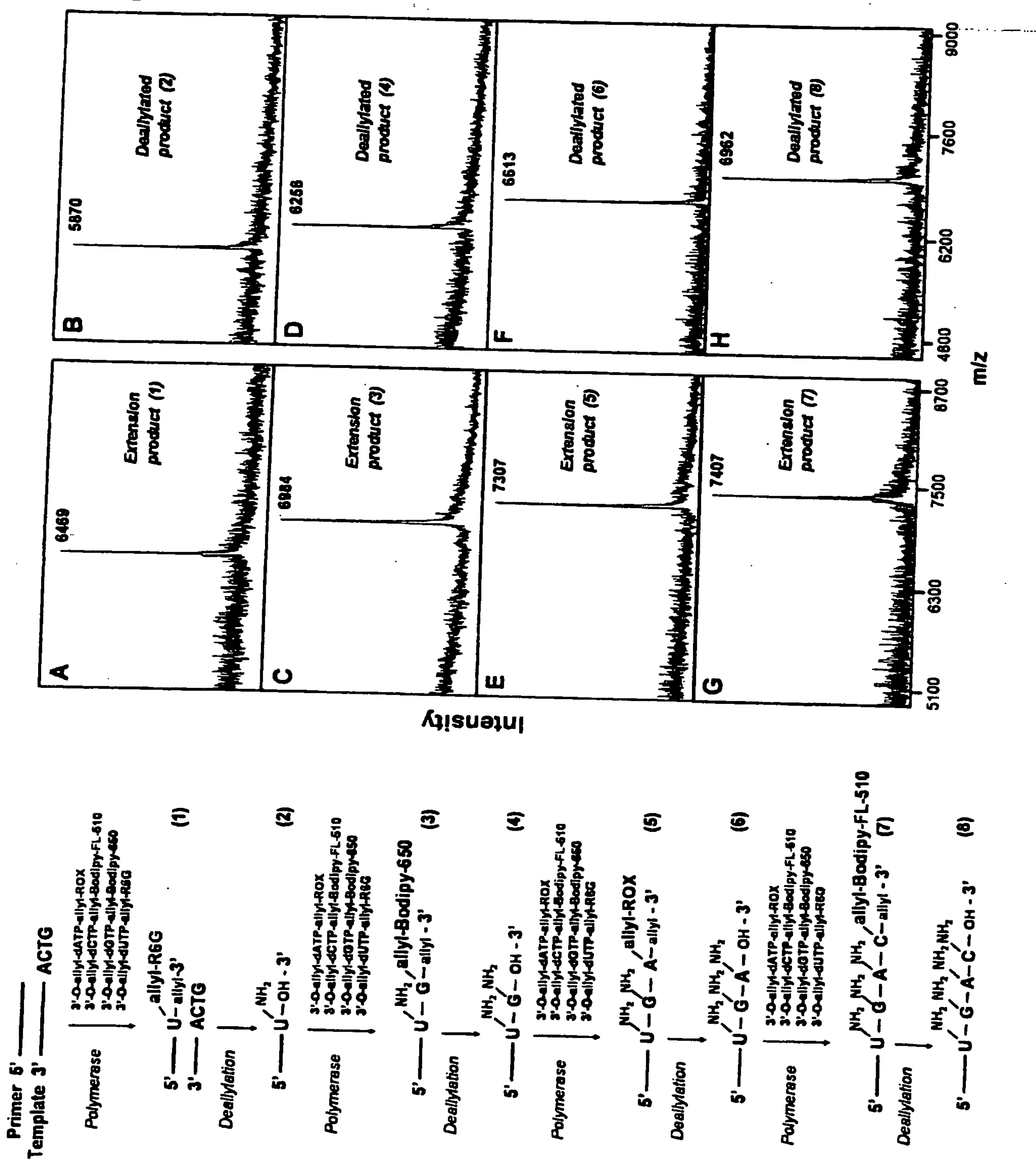

Primer 5'
Template 3' ACTG
Polymerase
3'-O-allyl-dATP-allyl-ROX
3'-O-allyl-dCTP-allyl-Bodipy-FL-510
3'-O-allyl-dGTP-allyl-Bodipy-650
3'-O-allyl-dUTP-allyl-R6G
allyl-R6G
5' — U — allyl-3'
3' — ACTG
(1)
Deallylation
5' — U — OH - 3'
(2)
5' — U — G — allyl - 3'
allyl-Bodipy-650
(3)
5' — U — G — OH - 3'
(4)
5' — U — G — A — allyl - 3'
allyl-ROX
(5)
5' — U — G — A — OH - 3'
(6)
5' — U — G — A — C — allyl - 3'
allyl-Bodipy-FL-510
(7)
5' — U — G — A — C — OH - 3'
(8)
NH_2
A
C
E
G
B
D
F
H
6469
6884
7307
7407
5870
6256
6613
6962
Extension product (1)
Extension product (3)
Extension product (5)
Extension product (7)
Deallylated product (2)
Deallylated product (4)
Deallylated product (6)
Deallylated product (8)
Intensity
m/z
5100
6300
7500
8700
4800
6200
7600
9000

Figure 4

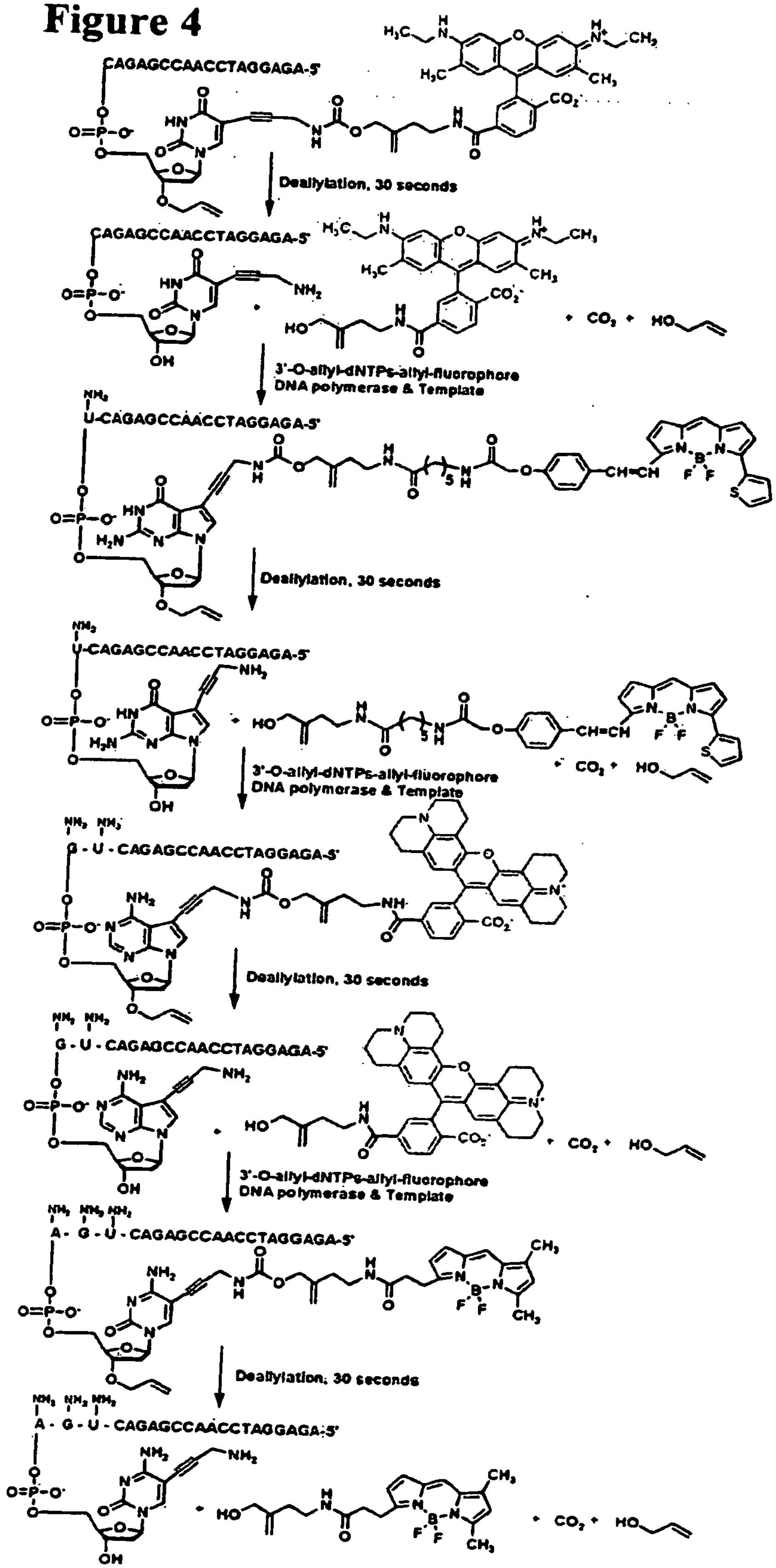

CAGAGCCAACCTAGGAGA-5'
Deallylation, 30 seconds
3'-O-allyl-dNTPs-allyl-fluorophore
DNA polymerase & Template
U-CAGAGCCAACCTAGGAGA-5'
Deallylation, 30 seconds
3'-O-allyl-dNTPs-allyl-fluorophore
DNA polymerase & Template
G - U - CAGAGCCAACCTAGGAGA-5'
Deallylation, 30 seconds
3'-O-allyl-dNTPs-allyl-fluorophore
DNA polymerase & Template
A - G - U - CAGAGCCAACCTAGGAGA-5'
Deallylation, 30 seconds

Figure 5

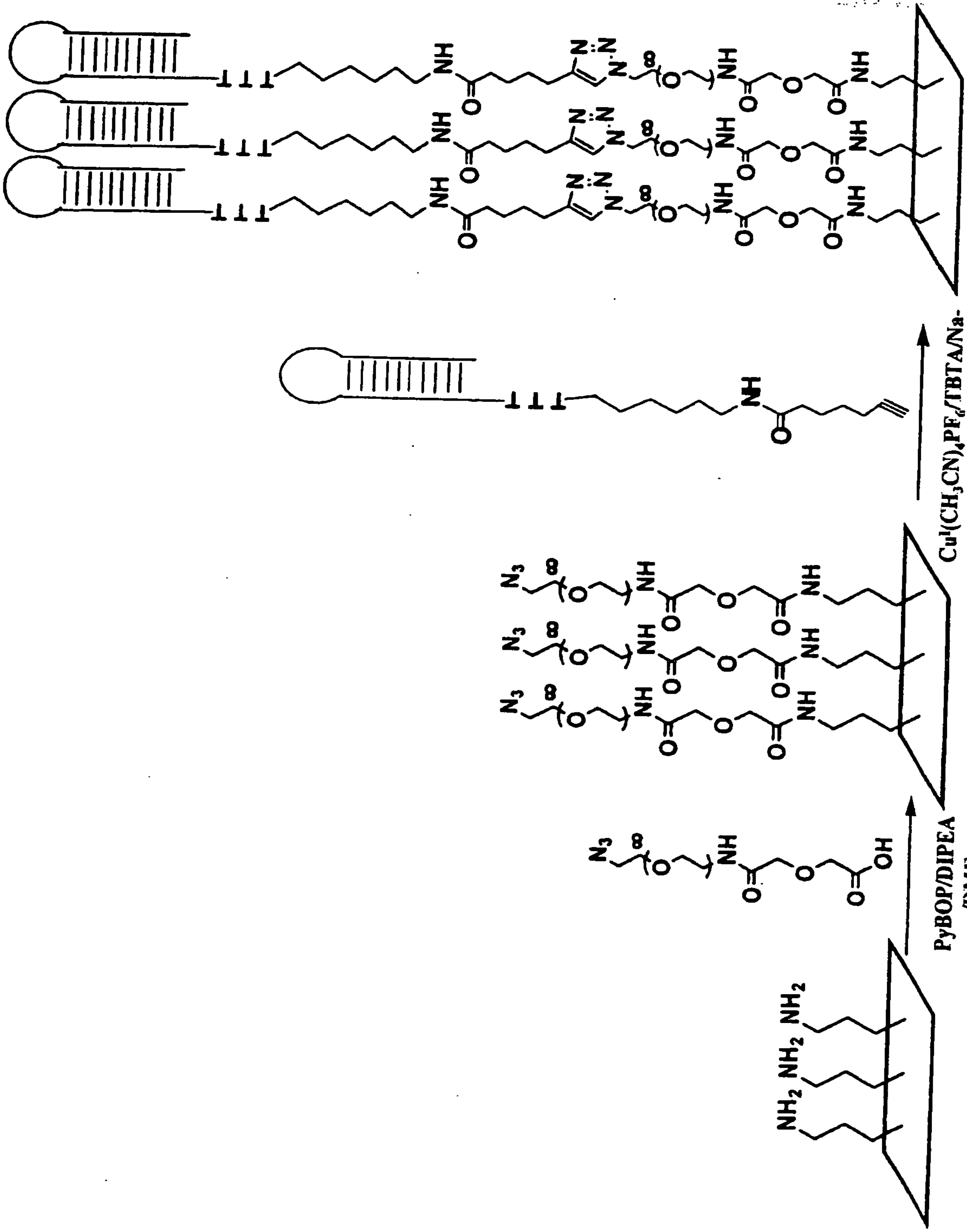


Figure 6

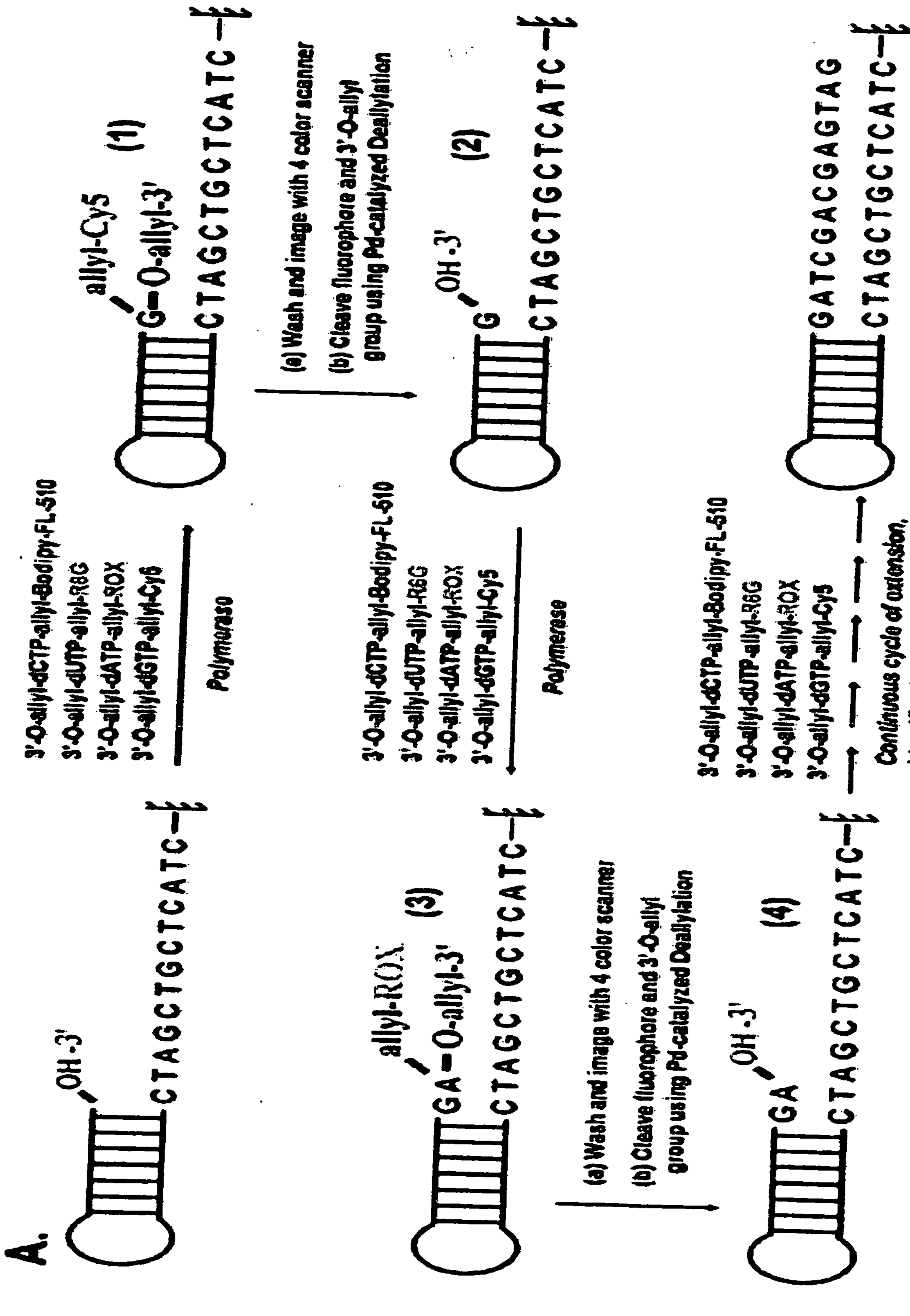

A.
OH-3'
CTAGCTGCTCATC
3'-O-allyl-dCTP-allyl-Bodipy-FL-510
3'-O-allyl-dUTP-allyl-R6G
3'-O-allyl-dATP-allyl-ROX
3'-O-allyl-dGTP-allyl-Cy5
Polymerase
allyl-Cy5
G-O-allyl-3'
CTAGCTGCTCATC
(1)
(a) Wash and image with 4 color scanner
(b) Cleave fluorophore and 3'-O-allyl group using Pd-catalyzed Deallylation
OH-3'
G
CTAGCTGCTCATC
(2)
3'-O-allyl-dCTP-allyl-Bodipy-FL-510
3'-O-allyl-dUTP-allyl-R6G
3'-O-allyl-dATP-allyl-ROX
3'-O-allyl-dGTP-allyl-Cy5
Polymerase
allyl-ROX
GA-O-allyl-3'
CTAGCTGCTCATC
(3)
(a) Wash and image with 4 color scanner
(b) Cleave fluorophore and 3'-O-allyl group using Pd-catalyzed Deallylation
OH-3'
GA
CTAGCTGCTCATC
(4)
3'-O-allyl-dCTP-allyl-Bodipy-FL-510
3'-O-allyl-dUTP-allyl-R6G
3'-O-allyl-dATP-allyl-ROX
3'-O-allyl-dGTP-allyl-Cy5
Continuous cycle of extension, identification, and cleavage
GATCGACGAGTAG
CTAGCTGCTCATC

Figure 6

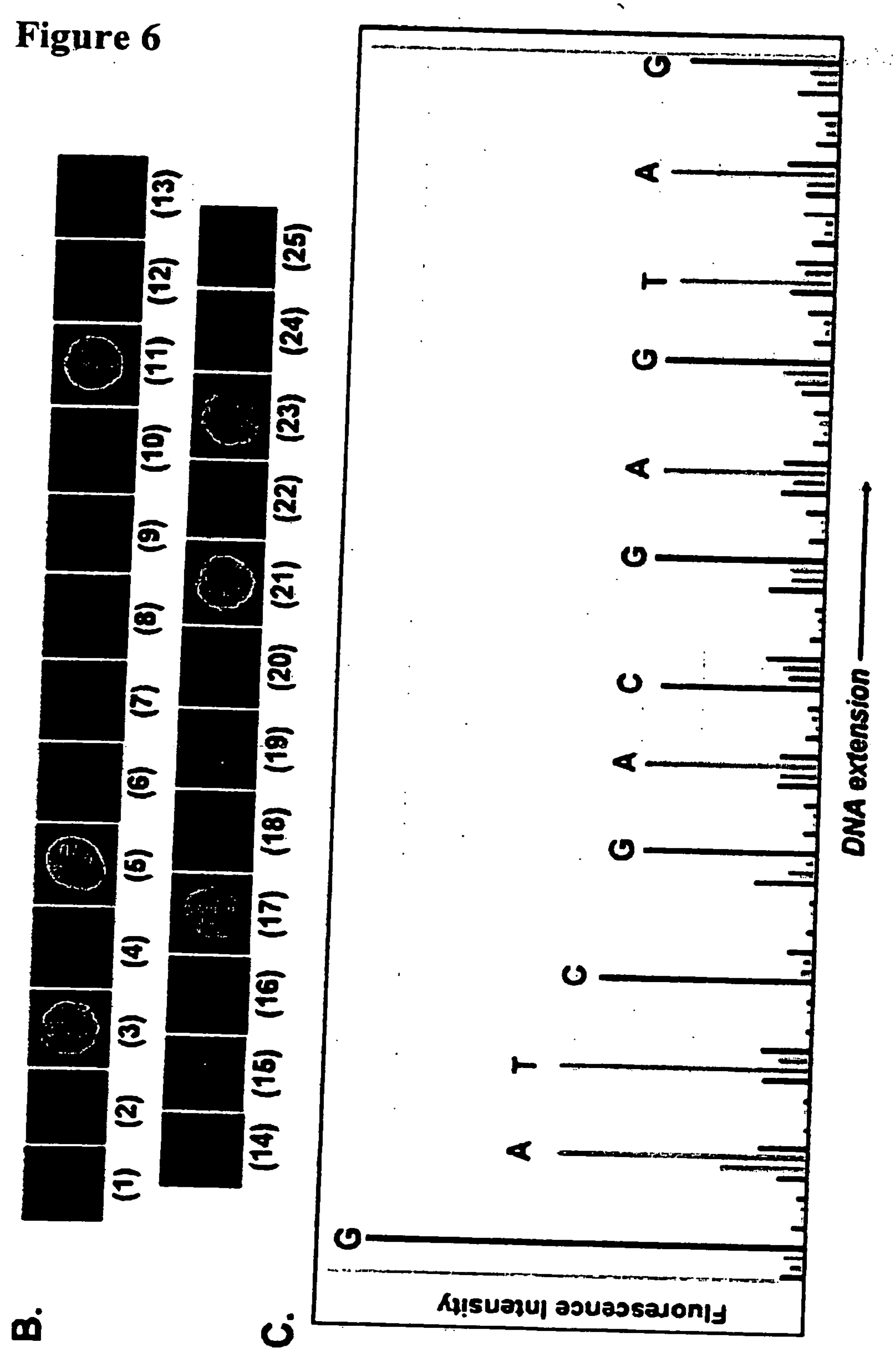

B.
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
(16)
(17)
(18)
(19)
(20)
(21)
(22)
(23)
(24)
(25)
C.
G
A
T
C
G
A
C
G
A
G
T
A
G
Fluorescence Intensity
DNA extension

Figure 7

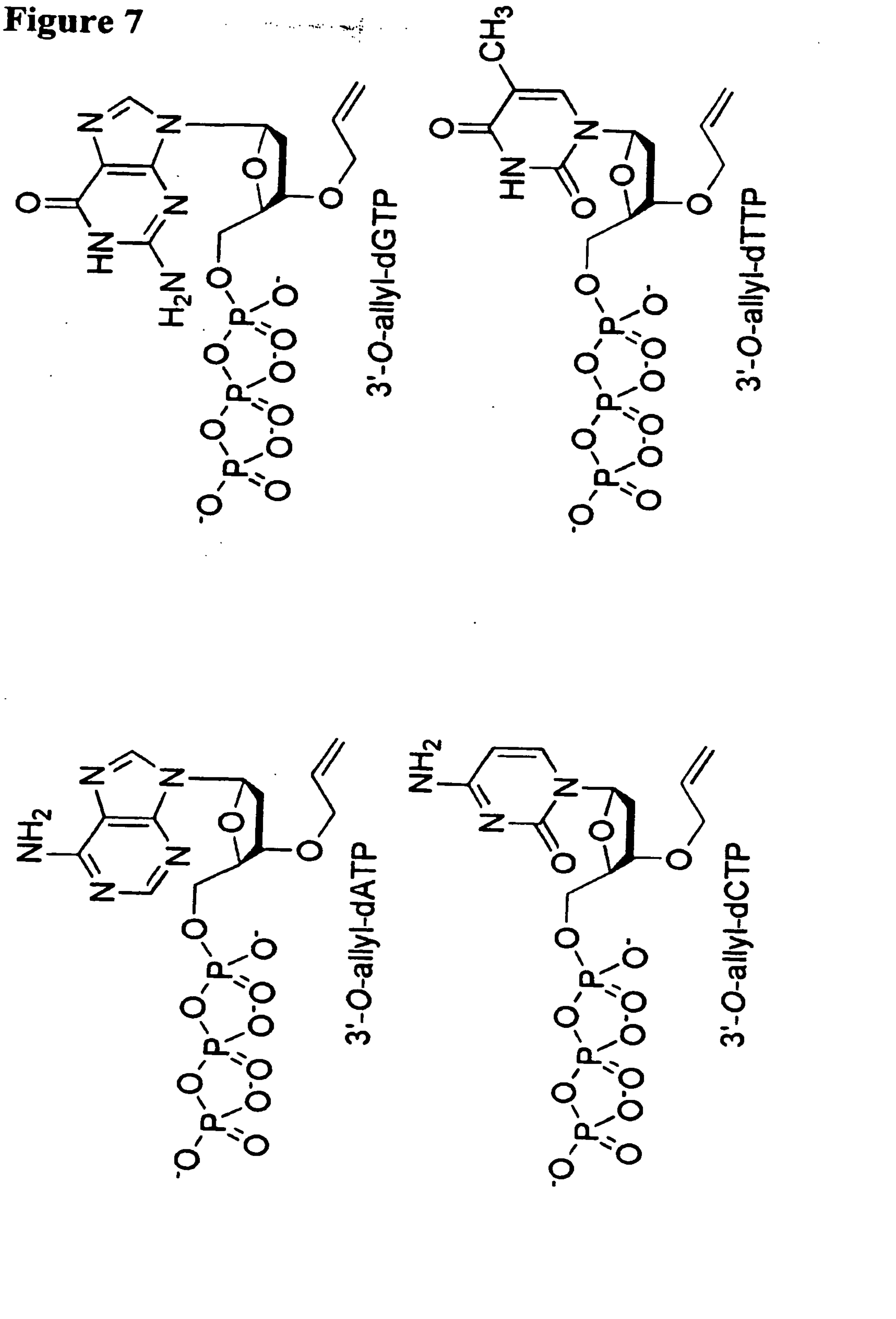


Figure 8

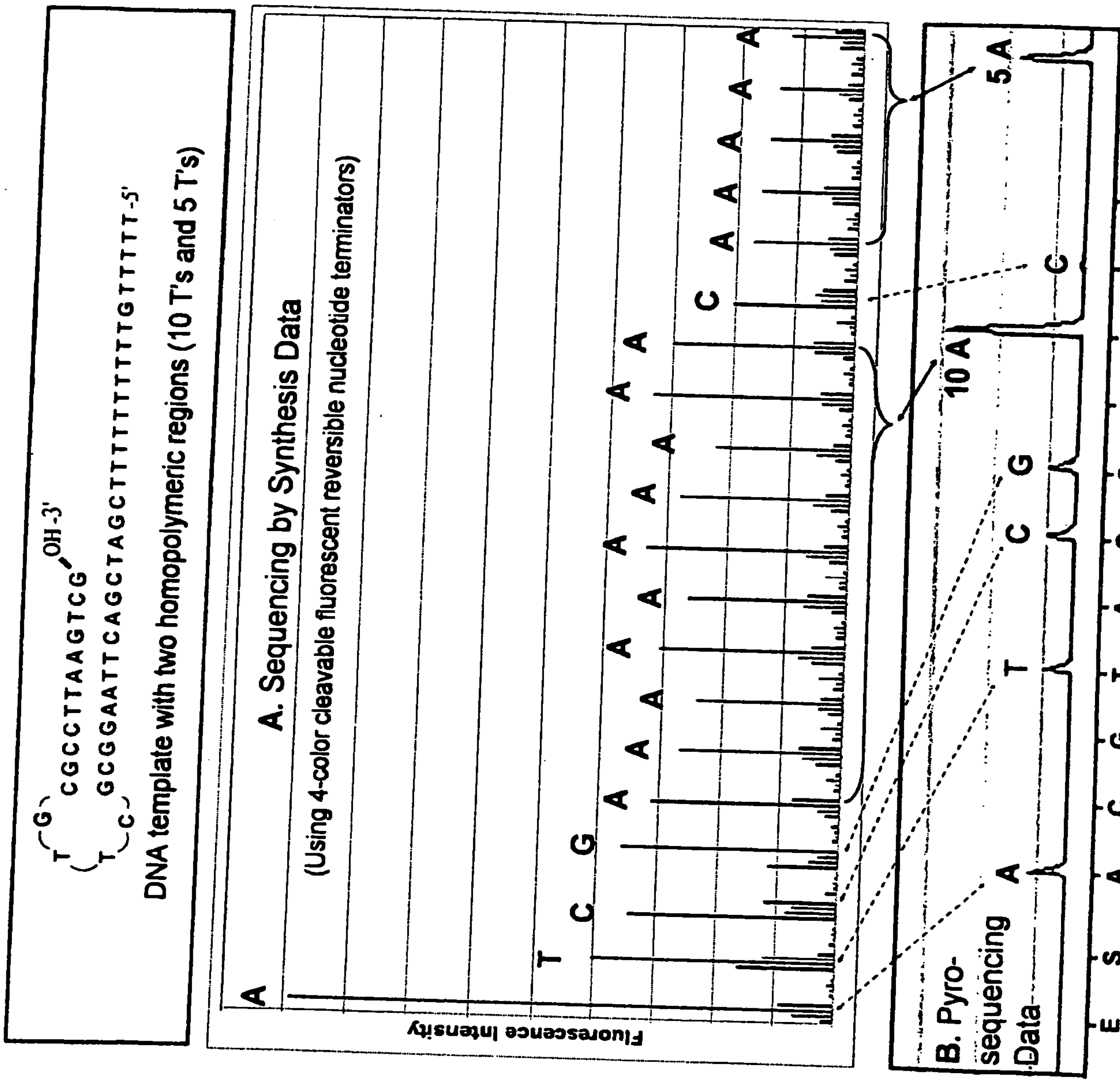

CGCCTTAAGTCG OH-3'
GCGGAATTCAGCTAGCTTTTTTTTTTGTTTTT-5'
DNA template with two homopolymeric regions (10 T's and 5 T's)
A. Sequencing by Synthesis Data
(Using 4-color cleavable fluorescent reversible nucleotide terminators)
Fluorescence Intensity
10 A
5 A
B. Pyro-sequencing Data
E S A C G T A C G T A C G T A

Figure 9

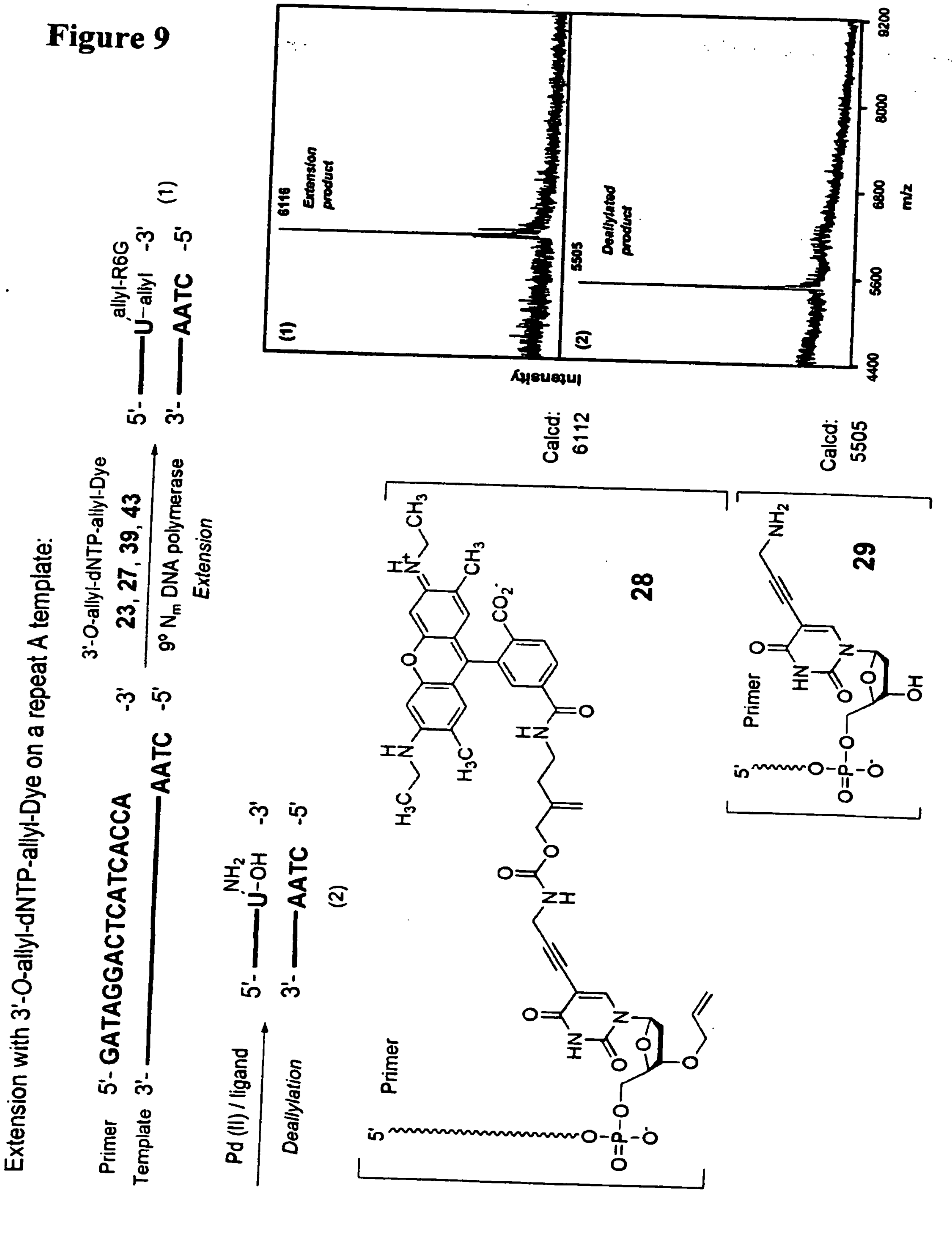
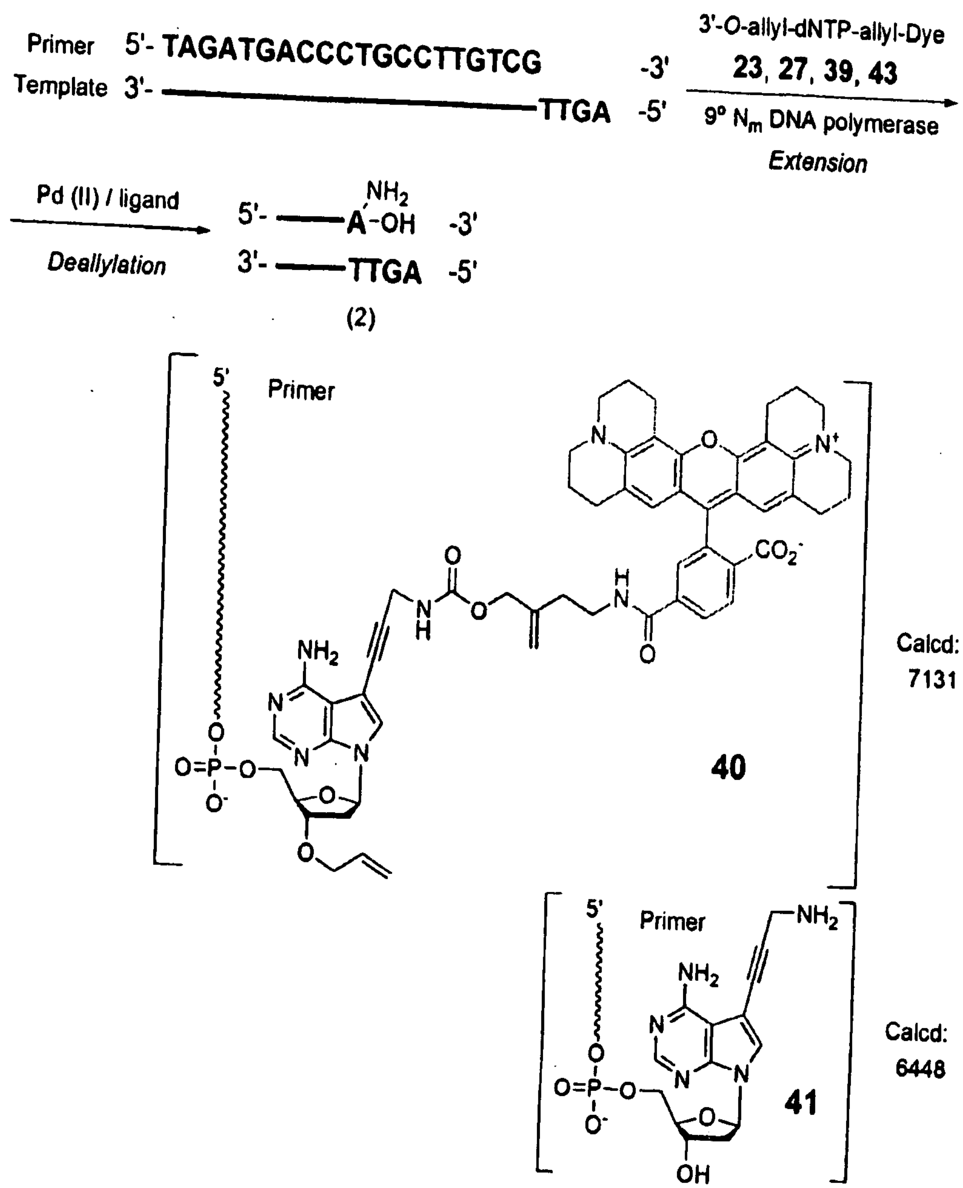

Extension with 3'-O-allyl-dNTP-allyl-Dye on a repeat A template:
Primer 5'- GATAGGACTCATCACCA -3'
Template 3'- AATC -5'
3'-O-allyl-dNTP-allyl-Dye
23, 27, 39, 43
9° N_m DNA polymerase
Extension
allyl-R6G
5'- U-allyl -3'
3'- AATC -5'
(1)
Pd (II) / ligand
Deallylation
NH2
5'- U-OH -3'
3'- AATC -5'
(2)
Primer
28
29
Calcd: 6112
Calcd: 5505
(1)
6116
Extension product
(2)
5505
Deallylated product
Intensity
4400
5600
6800
8000
9200
m/z

Figure 10

Extension with 3'-O-allyl-dNTP-allyl-Dye on a repeat T template:



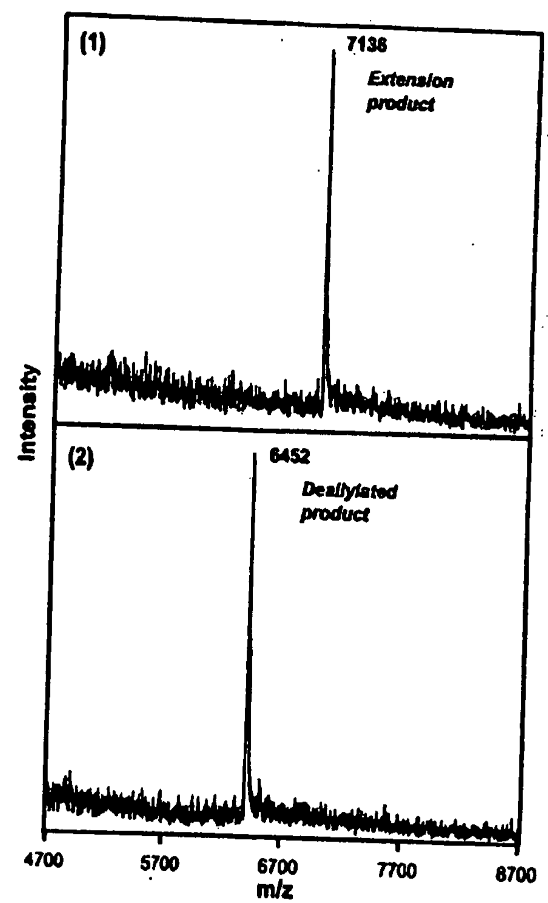
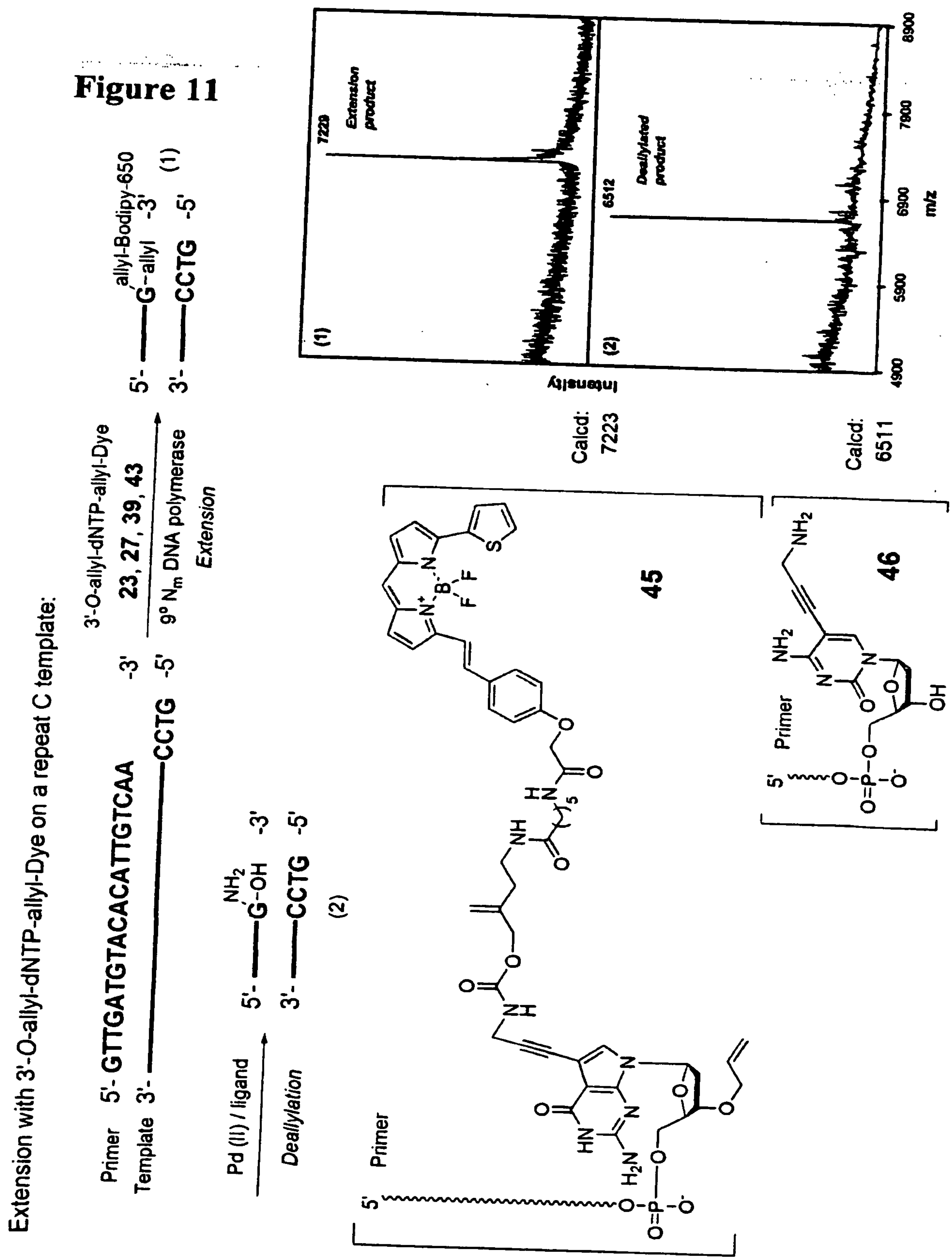


Figure 11


Extension with 3'-O-allyl-dNTP-allyl-Dye on a repeat C template:
Primer 5'- GTTGATGTACACATTGTCAA -3'
Template 3'- CCTG -5'
3'-O-allyl-dNTP-allyl-Dye
23, 27, 39, 43
9° Nm DNA polymerase
Extension
allyl-Bodipy-650
5'- G-allyl -3'
3'- CCTG -5'
(1)
Pd (II) / ligand
Deallylation
NH2
5'- G-OH -3'
3'- CCTG -5'
(2)
Primer
5'
45
46
Calcd: 7223
Calcd: 6511
Intensity
(1)
7229
Extension product
(2)
6512
Deallylated product
4900
5900
6900
7900
8900
m/z